Studies on Highly Sensitive Enzyme Immunoassays by Protein Chemistry(Dissertation_全文)

AUTHOR(S):

Morimoto, Koichi

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Abbreviations

ABTS	2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)
ALP	calf intestinal alkaline phosphatase
BSA	bovine serum albumin
bsmAb	bispecific monoclonal antibody
CA19-9	carbohydrate antigen 19-9
CEA	carcinoembrionic antigen
DEAE	diethylaminoethyl
DMEM	Dulbecco's modified Eagle's medium
DTNB	5,5'-dithio-bis(2-nitrobenzonic acid)
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
HPLC	high-performance liquid chromatography
IgG	immunoglobulin G
IgM	immunoglobulin M
IL-6	interleukin-6
mAb	monoclonal antibody
PBS	phosphate-buffered saline
pNPP	*p*-nitrophenyl phosphate
SDS-PAGE	sodium dodesylsulfate-polyacrylamide gel electrophoresis
sialyl Lewis [a]	sialylated lacto-*N*-fucopentaose II

sELISA	solid-phase sandwich enzyme-linked immunosorbent assay
SAMSA	*S*-acetylmercapto-succinic anhydride
SPDP	*N*-succinimidyl-3-(2'-pyridyldithio)propionate
TNB	thionitrobenzonic acid
Tris	tris(hydroxymethyl)aminomethane
TSH	thyroid-stimulating hormone

General Introduction

Antibodies are key molecules in host immune system. They are produced in B lymphocytes and are composed of five kinds of structurally similar molecules (IgG, IgM, IgA, IgD, and IgE). They usually have rather high binding affinities for their target substances (antigens) by sites on the Fab region, and they have numerous biological effector functions mediating the immune response subsequent to binding antigen with sites on the Fc region.

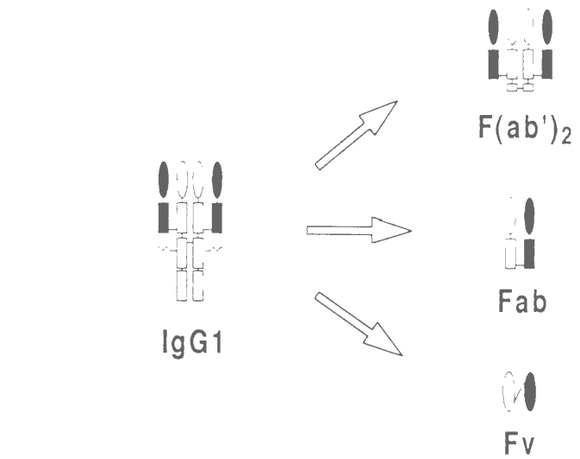
The invention of monoclonal antibody (mAb) technology (1) has provided preparation of homogeneous antibodies. A single clone of a B cell from the spleen of an immunized animal, mouse or rat for most cases, is fused with a mouse myeloma cell, and the resulting hybridoma produces antibodies of a single specificity. mAbs are widely used *in vitro* diagnosis (blood, serum, urine, and sputum), *in vivo* diagnosis (cell imaging), therapy (drug targeting and T-cell targeting), and purification procedure (affinity chromatography) (2-4). They also start to be used even in chemical reaction as catalysts (5). For *in vitro* diagnosis, enzyme-linked immunosorbent assays (ELISA) (6), radioimmunoassays (7), immunocytopathology, and flow cytometric analysis are main targets to which antibodies are applied (8).

The binding affinity of antibodies against antigen is so strong that wide varieties of *in vitro* diagnosis have been established and tested. (a) Agglutination: antibodies bind to cell surface antigen on bacterium, red blood cell, and leukocyte. It is well known hemagglutinin and latex fixation. (b) Immunodiffusion: antibodies bind the specific antigens (protein and polysaccharide) to an insoluble immunocomplex and precipitate in agar gel (Ouchterlony diffusion and Oudin diffusion). (c) Immunoassay: antibody or

antigen, which was conjugated with radioisotope (radioimmunoassay) (7), fluorescent probe (fluoroimmunoassay), and enzyme (enzyme immunoassay) (6) bind antigen or antibody in soluble matrix. The immunocomplex can be detected by radiation dose, fluorescent intensity, or enzyme activity, respectively. *In vitro* diagnosis have been significantly developed and constantly updated to detect less concentration of antigen.

Active fragments of antibodies are easier to be manipulated for industrial application than the original mAbs, because they do not retain any biological functions due to Fc regions (9, 10), and the interaction of mAbs with non-specific proteins (complement, rheumatoid factor, and anti-animal antibody) is reduced. Having a smaller molecular mass than the mAbs is advantageous, and preparation of $F(ab')_2$ fragments by protease digestion of mouse mAbs have been reported (11-13). Active fragments of an antibody (Fv, Fab, $F(ab')_2$, and $F(ab')_{2\mu}$; see Fig. 1) are usually prepared by biochemical cleavage. This study is focused on the purification and application of $F(ab')_2$ fragments, most commonly used antigen-binding fragments, of high yields and in large-scale. In Chapter 1, single-step purification of $F(ab')_2$ fragments of mAbs (immunoglobulins G1 and M) by hydrophobic interaction high-performance liquid chromatography was described. Especially, the $F(ab')_2$ fragments from IgM was prepared first in this study, and was named $F(ab')_{2\mu}$ fragments. Preparation of $F(ab')_{2\mu}$ fragments from rat IgM monoclonal antibodies was also described and they were applied to the enzyme immunoassay of mouse interleukin-6. In Chapter 2, flow cytometric analysis of sialyl Lewis ^{a} antigen on human cancer cells by using $F(ab')_{2\mu}$ fragments prepared from a mouse IgM monoclonal antibody was described.

Bispecific $F(ab')_2$ fragments can bind to two different antigens simultaneously , and have potential uses for wide application (14-18). The author developed a new ELISA system using bispecific $F(ab')_2$ fragments with polymerized alkaline phosphatase. In Chapter 3, a



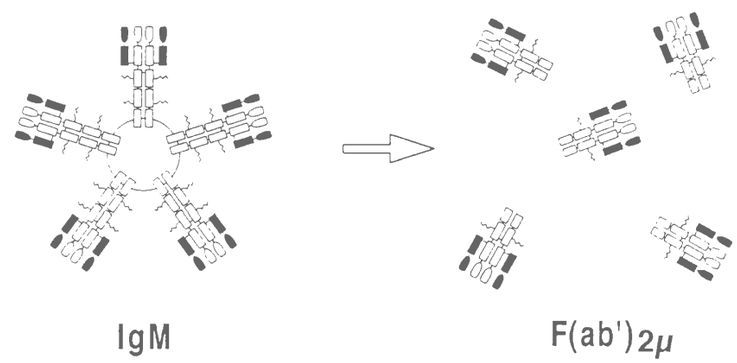


Fig. 1. Fragmentation patterns of murine IgG1 and IgM.

sensitive enzyme immunoassay of thyroid-stimulating hormone (TSH) by using bispecific $F(ab')_2$ fragments recognizing polymerized alkaline phosphatase and TSH is described.

Finally, effects of blocking conditions with bovine serum albumin on enzyme immunoassay were examined (Chapter 4). A binding-activity of immobilized monoclonal antibody in acidic conditions (100 mM citrate buffer, pH 3.5) increases and non-specific binding is reduced.

Some problems may be overcome by the use of $F(ab')_2$, $F(ab')_{2\mu}$, and bispecific $F(ab')_2$ fragments prepared in this study and that highly sensitive enzyme immunoassay can be achieved by protein chemistry.

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Chapter 1

Preparation of Active Fragments of Immunoglobulin and their Application to Enzyme-linked Immunosorbent Assay

Single-step Purification of $F(ab')_2$ Fragments of Mouse Monoclonal Antibodies (Immunoglobulins G1) by Hydrophobic Interaction High Performance Liquid Chromatography

Introduction

It is well known that mouse $F(ab')_2$ fragments are more useful than the original whole mAbs because they do not retain any biological functions due to Fc regions and interaction with non-specific binding is reduced. Some papers have reported the preparation of $F(ab')_2$ fragments by pepsin digestion of mouse IgG1 mAbs (1) Generally, digestion proceed for 12-48 h at the weight ratio, IgG1/pepsin= 40-100, and $F(ab')_2$ fragments were purified by size exclusion chromatography (2), ion-exchange chromatography (3, 4) or protein A-Sepharose chromatography (4). However, these methods are time-consuming, and can not afford sufficient purity and recovery of $F(ab')_2$ fragments. Development of efficient procedures for $F(ab')_2$ fragments preparation is an urgent necessity.

In this chapter, the author describes the single-step purification of $F(ab')_2$ fragments from pepsin digests of mAbs (IgG1 isotype) by hydrophobic interaction HPLC using TSKgel Phenyl-5PW. The author shows that this method is suitable for the large-scale purification of $F(ab')_2$ fragments

Materials and Methods

mAbs

Five mouse mAbs of the IgG1 class were used. CU203.2, EM89.6, FS42.7, FE138 and GC4. Their specific antigens are, respectively, human carcinoembryonic antigen (CEA) human myoglobin, human follicle stimulating hormone, human IgE and human growth hormone. The hybridomas secreting these mAbs were established in the laboratory to which the author belongs (unpublished data), by fusing spleen cells from an antigen-immunized BALB/c mouse with NS-1 myeloma cells according to Köhler and Milstein (5). The hybridoma cells were injected into pristane-primed BALB/c mice, and were grown in ascites fluids (6). mAbs were purified from the ascites at 4°C. The collected ascites were centrifuged at 3,000 x g for 20 min to remove cells, and the supernatants were passed through Millipore filters (pore size: 0.8 μm pore; AA type). Ammonium sulfate (solid) was added to the filtrate to give 50% saturation, followed by centrifugation at 10,000 x g after standing for 2 h, The precipitate were dissolved in PBS, pH 7.4. Ammonium sulfate (solid) was added to give 50% saturation, and the precipitates were collected by centrifugation as above, and dissolved in 100 mM citrate buffer (pH 3.5) to give a protein concentration of 10-20 mg/ml. Undissolved materials were removed by centrifugation at 10,000 x g for 20 min. This centrifugation step was critical in reducing the period of pepsin digestion (see Discussion). The supernatant was adjusted to pH 3.7 with 1 M HCl or NaOH, giving partially purified mAb.

Pepsin digestion

The partially purified mAb was digested by porcine pepsin (EC 3.4.23.1), (Lot No. 117F-8080; 3900 U/mg according to the supplier) purchased from Sigma (St. Louis, MO). The starting concentration of mAb was 10-20 mg/ml in 100 mM citrate buffer (pH 3.5). Pepsin was added to the mAb solution at a weight ratio of 1:100 (pepsin : IgG1). Digestion proceeded with gentle stirring at 37°C for 2 h, and was stopped by adding 3 M tris (hydroxymethyl)aminomethane (Tris) to give a pH around 7.

Analytical studies

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a 12% and 16% slab gel under reducing conditions and a 6% slab gel under non-reducing conditions according to the method of Laemmli (7). Proteins were reduced by treatment with 2.5% 2-mercaptoethanol at 100°C for 10 min. Proteins were stained with Coomassie brilliant blue R-250. The molecular mass marker kit consisting rabbit muscle phosphorylase b (94 kDa), bovine serum albumin (BSA) (67 kDa), chicken ovalbumin (43 kDa), bovine erythrocyte carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and bovine milk α-lactalbumin (14.2 kDa) is a product of Pharmacia (Uppsala, Sweden).

Concentrations of $F(ab')_2$ fragments and mAb were estimated using an extinction coefficient at 280 nm, A (1 mg/ml)=1.4 (8).

Antigen binding activities of mAb and $F(ab')_2$ fragments were measured by a solid-phase enzyme immunoassay (9). A microtiter plate (96 wells; Nunc-Intermed, MaxiSorp; Roskilde, Denmark) was coated with the antigen by addition of 100 µl antigen solution of various concentrations (2.0, 1.0, 0.5, 0.25, 0.125, and 0 µg/ml) in 50 mM carbonate buffer (pH 9.5) to each well, and incubated for 1 h at 37°C. The plate was blocked by incubation with 0.2% BSA in PBS overnight at 4°C, washed with PBS, and incubated with mAb or

$F(ab')_2$ for 1 h at 37°C. After washing once more with PBS, excess goat anti-mouse IgG ($F(ab')_2$ fragment specific) antibody conjugated with horseradish peroxidase (1/5,000; Tago Inc., Burlingame, CA) was added to the plate and incubated for 1 h at 37°C. The plate was washed with PBS, then the enzyme reaction was started by adding 100 µl substrate (0.03% 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 0.03% H_2O_2 in 100 mM citrate buffer, pH 4.1) to each well, and then terminated by 100 µl oxalic acid (0.1 M) after reaction for 5 min at 25°C. The absorbance at 415 nm was measured by a microtiter plate reader MPR-A4 (Tosoh, Tokyo, Japan).

High performance liquid chromatography

The HPLC apparatus composed of a solvent-delivery system CCPM, a UV monitoring system UV8010, a fraction collector FC8000 and a computer control system SC8010 was purchased from Tosoh. Throughout this section, the elution was monitored by absorbance at 280 nm, and each fraction (1 ml) were collected.

Diethylaminoethyl HPLC (DEAE-HPLC) was performed on a TSKgel DEAE-5PW column (7.5 mm (inner diameter) x 75 mm) (Tosoh), equilibrated with staring buffer, 20 mM Tris-HCl (pH 8.0) containing 40 mM NaCl. The pepsin digests were dialyzed against the starting buffer, and applied to the column. A linear gradient of NaCl from 40 to 500 mM in the same buffer was generated in 30 min at a flow-rate of 1 ml/min.

Hydrophobic interaction HPLC was performed on a TSKgel Phenyl-5PW column (7.5 mm (inner diameter) x 75 mm) (Tosoh). Pepsin digests of mAbs were salted out with 60% saturated ammonium sulfate, and the precipitates were immediately dissolved in PBS containing 1 M ammonium sulfate (pH 7.4). The solution was applied to the column equilibrated with the same buffer, and eluted with a linear gradient of ammonium sulfate

from 1 to 0 M in PBS (pH 7.4), for 30 min at a flow-rate of 1 ml/min at room temperature.

Gel filtration HPLC was performed using a TSKgel G3000SW$_{XL}$ (7.8 mm (inner diameter) x 30 cm) (Tosoh) with 50 mM phosphate buffer containing 150 mM sodium sulfate (pH 6.5) at a flow-rate of 1 ml/min.

Results

Pepsin digestion

Time dependence of pepsin digestion of IgG1 (CU203.2; mouse anti- CEA mAb), was monitored by SDS-PAGE (Fig. 1A and B). After the incubation time indicated in Fig. 4, the reaction was stopped. Half of the reaction mixture was applied to SDS-PAGE (6% gel) under non-reducing conditions (Fig. 1A), and the other half to SDS-PAGE (12% gel) under reducing conditions after incubation with 2-mercaptoethanol (Fig. 1B). The ascites fluids, from which the mAb was partially purified by ammonium sulfate, were also applied to SDS-PAGE under both conditions. As shown in Fig. 1A, a 160 kDa band corresponding to IgG disappeared completely after digestion for 120 min. On the other hand, a 110 kDa band which is considered to be F(ab')$_2$ fragments appeared during the reaction. Under reducing conditions (Fig. 1B), IgG shows two bands before the reaction, of 50 kDa and 28 kDa, which correspond to heavy (H) and light (L) chains, respectively. The H chain disappeared in the reaction, and a new band of 30 kDa appeared. The L chain appeared not to be degraded in the reaction. After a 2-h digestion, the H chain band disappeared completely, and only 30 kDa and 28 kDa bands remained. The new 30 kDa band must be derived from cleavage of the H chain.

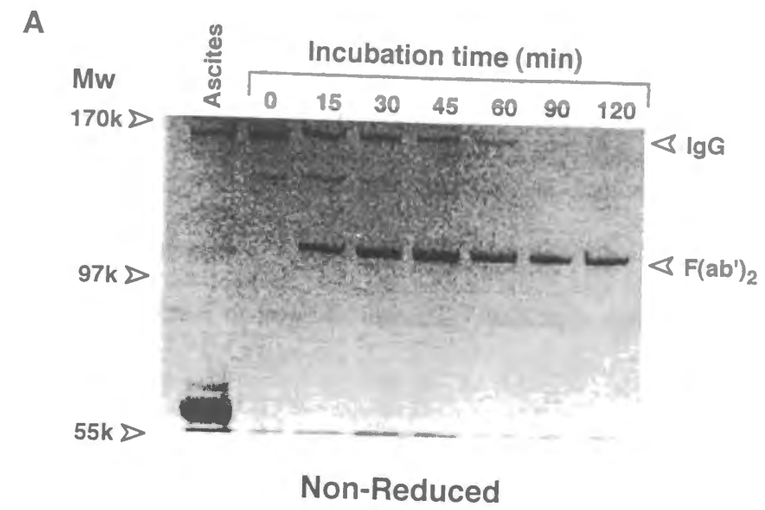


Fig. 1. SDS-PAGE of mouse mAb CU203.2 (IgG1) digested with pepsin at the pepsin : mAb ratio of 1 : 100 (w/w) at 37°C in 100 mM citrate buffer, pH 3.7, fo r the various incubation times indicated. Panel A: non-reducing conditions; the digests were applied to SDS-PAGE without 2-mercaptoethanol treatment. Panel B: reducing conditions; prior to SDS-PAGE, the digests were treated with 2.5% 2-mercaptoethanol at 100°C for 10 min.

Similar time courses of pepsin digestion were obtained for four other mAbs (IgG1) examined (data not shown). Throughout this study, mAb solutions were prepared from mouse ascites fluids by centrifugation at 10,000 x g for 20 min after precipitation by 50% saturated ammonium sulfate and dissolution of the precipitates with 100 mM citrate buffer (pH 3.5). The mAb solutions prepared without centrifugation required up to 12 h for completion of the pepsin digestion. The centrifugation is critically significant in reducing the digestion time.

Purification of F(ab')$_2$ fragments by DEAE-HPLC

Products of the pepsin digestion for 2 h were separated by DEAE-HPLC using a TSKgel DEAE-5PW column, and fractions (1 ml each) were collected every 1 min. An elution pattern for pepsin digests of mAb CU203.2 is shown in Fig. 2. F(ab')$_2$ fragments were separated clearly from intact mAbs (elution time: 26 min), pepsin and other peptides were also eluted in the void volume (8, 10). In fact, the fractions eluted in the void volume were heterogeneous in protein composition as verified by SDS-PAGE (data not shown). This suggests that DEAE-HPLC is not sufficient for purification of F(ab')$_2$ fragments and that further purification steps are required.

Hydrophobic interaction HPLC

The pepsin digests were separated by hydrophobic interaction HPLC using a TSKgel Phenyl-5PW column. Chromatograms for the pepsin digests of mAb CU203.2 obtained by the digestion for 0, 15, and 120 min are shown in Fig. 3. A linear gradient of ammonium sulfate from 1 to 0 M was generated over 30 min, and the elution was continued using ammonium sulfate free PBS. The mAb was eluted at 34 min, and as pepsin digestion

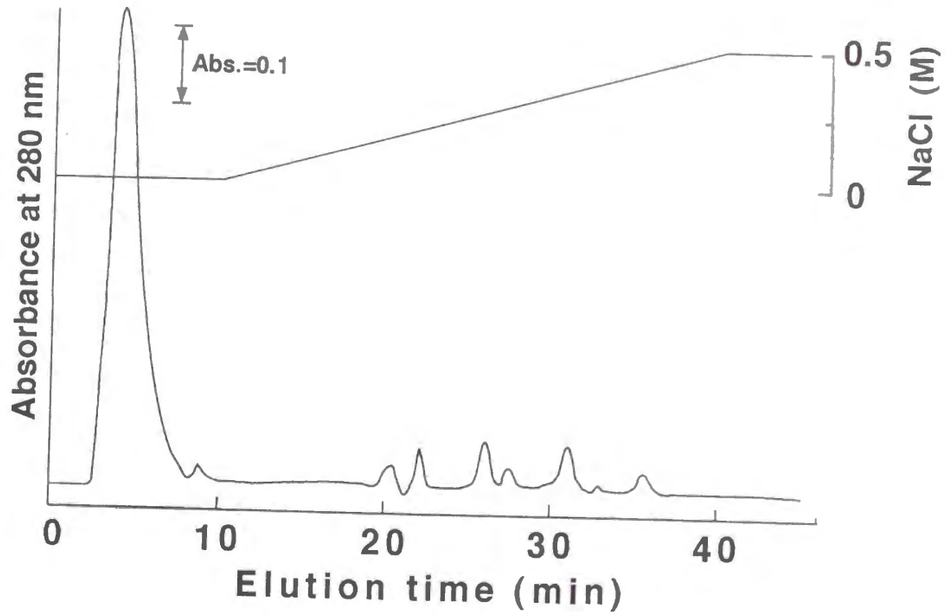


Fig. 2. Purification of F(ab')$_2$ fragments from pepsin digests of mAb CU203.2 by anion exchange HPLC on a TSKgel DEAE-5PW column. The digests were prepared by incubation for 120 min under the conditions.

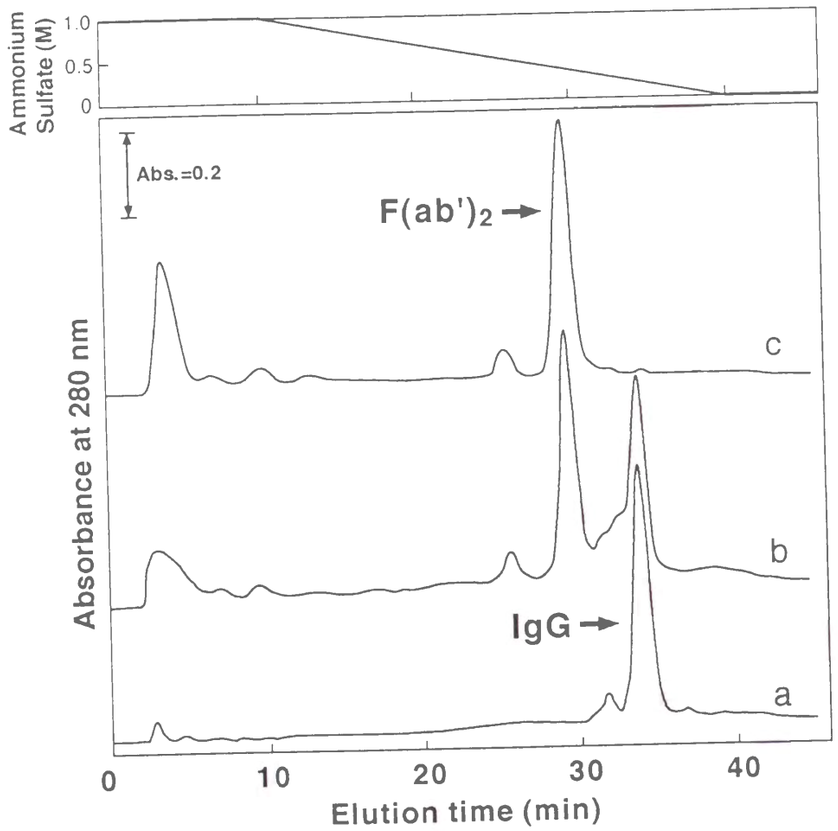


Fig. 3. Purification of $F(ab')_2$ fragments from pepsin digests of mAb CU203.2 by hydrophobic interaction HPLC on a TSKgel Phenyl-5PW column. Incubation time with pepsin: a, 0 min; b, 15 min; c, 120 min.

progressed, the mAb peak decreased. The peak disappeared completely after digestion for 120 min, and inversely, a new peak corresponding to $F(ab')_2$ fragments appeared at 30 min. Fractions from 28 to 31 min were collected for further analysis.

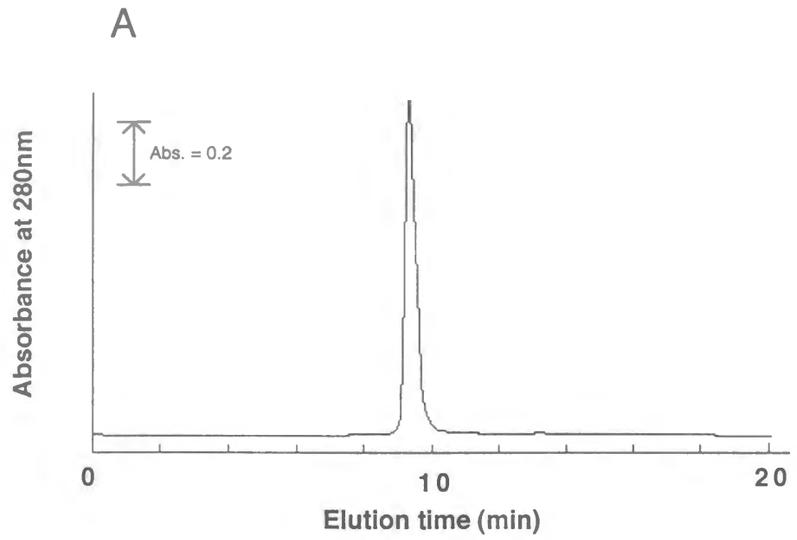
Purity of $F(ab')_2$ fragments

The fractions collected from 28 to 31 min in the hydrophobic interaction HPLC (Fig. 6) were applied to gel filtration HPLC on a TSKgel G3000SW$_{XL}$ column (Fig. 4A). The proteins were eluted as a single peak, showing that the purity of the $F(ab')_2$ fragments was more than 98%. SDS-PAGE (Fig. 4B) of the fraction showed only two bands corresponding to H and L chains (Fig. 4B, lane 2), and the contaminating proteins observed before the HPLC (Fig. 4B, lane 1) were absolutely removed.

The purification processes are summarized in Table 1. In the case of mAb CU203.2, the total protein in 10 ml of ascites fluids was 208 mg and that after ammonium sulfate precipitation, it was 24 mg, estimated from the mAb peak at the elution time of 8.6 min in the gel filtration HPLC (Fig. 4A). When the total mAb was theoretically entirely converted to $F(ab')_2$ by pepsin digestion, the ratio of $F(ab')_2$ should be two thirds of the mAb quantity, namely 16 mg. The quantity of the latter obtained from the hydrophobic interaction HPLC was 9 mg, thus, the yield of $F(ab')_2$ was 56%. The material balances for other mAbs are listed in Table 1. The mAb concentrations in the ascites fluids were all different. However, the purity of $F(ab')_2$ was greater than 98%, and the yield was in a narrow range, 42-58%.

Solid-phase enzyme immunoassay

Fig. 5 shows immunoreactivities of mAb CU203.2 and its $F(ab')_2$ fragments against the specific antigen, CEA. Each well of a microtiter plate was coated with 100 μl of CEA



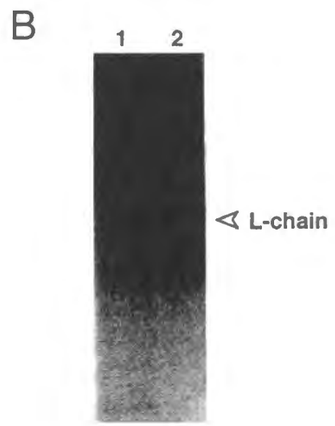


Fig. 4. Characterization of $F(ab')_2$ fragments purified from pepsin-digests of mAb CU203.2 by hydrophobic interaction HPLC on a TSKgel Phenyl-5PW column. (A) Analytical gel filtration HPLC on a TSKgel $G3000SW_{XL}$ column. (B) SDS-PAGE under reducing conditions. Lane 1, pepsin digests of mAb CU203.2; lane 2, purified fractions of $F(ab')_2$ fragments by the Phenyl-5PW HPLC.

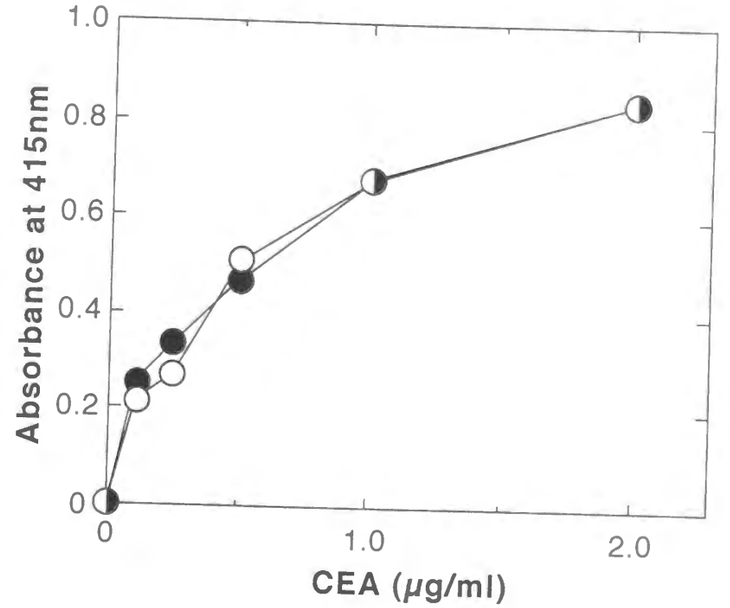


Fig. 5. Immunoreactivities of mAb CU203.2 and its $F(ab')_2$ fragment against their specific antigen, CEA. Each well of a 96-well microtiter plate was coated with CEA by adding 100 μl CEA at the concentrations shown in the horizontal axis. 100 μl of 12 nM mAb (●) or $F(ab')_2$ (○) was added, followed by goat anti-mouse IgG ($F(ab')_2$ fragment specific) antibodies conjugated with peroxidase. Absorbance at 415 nm generated by the reaction with ABTS for 5 min was observed.

Table 1 Purification of mouse $F(ab')_2$ fragments by hydrophobic interaction HPLC by TSKgel Phenyl-5PW

Monoclonal antibody	Volume of ascites (ml)	Protein in ascites (mg)	IgG for pepsin digestion [a] (mg)	$F(ab')_2$ purified [b] (mg)	Purity of $F(ab')_2$ (%)	Recovery yield of $F(ab')_2$ [c]
CU203.2	**10**	**208**	**24**	**9**	**98**	**0.56**
EM89.6	**10**	**304**	**199**	**56**	**98**	**0.42**
FS42.7	**250**	**5860**	**3000**	**938**	**99**	**0.47**
FE138	**180**	**6170**	**3520**	**1330**	**99**	**0.57**
GC4.5	**406**	**13020**	**5700**	**2200**	**98**	**0.58**

[a] Quantity of IgG obtained after precipitation of ascitic fluids with 50% saturated ammonium sulfate. The quantity was estimated by absorbance at 280 nm after gel filtration HPLC using TSKgel G3000SW XL .

[b] Quantity of $F(ab')_2$ fragments purified by hydrophobic interaction HPLC using TSKgel Phenyl-5PW. The quantity was estimated by absorbance at 280 nm.

[c] (Quantity of $F(ab')_2$ (mg)) / (Quantity of IgG1 (mg) x 2 / 3).

solutions at various concentrations; 2.0, 1.0, 0.5, 0.25, 0.125, and 0 μg/ml, in 100 mM carbonate buffer (pH 9.5). After blocking with BSA, 100 μl of the 12 nM mAb or $F(ab')_2$ fragments, namely 1.9 μg/ml mAb and 1.3 μg/ml $F(ab')_2$ fragments, respectively, was added. Their immunoreactivities against CEA were in good agreement, suggesting that the immunoreactivity of mAb CU203.2 is maintained entirely in the $F(ab')_2$ fragments.

Discussion

There have been some papers reporting the purification of $F(ab')_2$ fragments by gel filtration (2) or ion-exchange chromatography (3, 4), after pepsin digestion of IgG. However, $F(ab')_2$ fragments can not be purified to homogeneity by single-step chromatography using DEAE-HPLC (Fig. 2) in this section. The author confirmed that the $F(ab')_2$ fragments flowed through not only anion-exchange chromatography (TSKgel DEAE-5PW column) but also cation-exchange chromatography (TSKgel SP-5PW column) (data not shown).

In this section, the author described the application of hydrophobic interaction HPLC using TSKgel Phenyl-5PW to the single-step purification of $F(ab')_2$ fragments of mouse mAbs of the IgG1 isotype. Generally in this HPLC, proteins of interest are adsorbed on the resin by hydrophobic interaction at high salt concentration, and removed by decreasing the concentration. One of the features of this HPLC is that ammonium sulfate can be used as the salt for controlling the adsorption and removal of the proteins. Ammonium sulfate is widely used as a salting-out reagent in protein purification, because its effects on proteins are mild and the solubility is considerably high. In our experience, mAbs (IgG1) and $F(ab')_2$

fragments are stable in the presence of ammonium sulfate, although it has been reported that the immunoreactivity of a mAb was reduced significantly by precipitation with ammonium sulfate (11). The author showed in this section that $F(ab')_2$ fragments treated by 60% saturated ammonium sulfate, could be applied to hydrophobic interaction HPLC after adjusting the ammonium sulfate concentration to that of the starting buffer, namely 1 M.

The advantage of the purification of $F(ab')_2$ fragments described in this section is not only that the procedure takes only a single-step, but also that no buffer exchange, such as dialysis, is required. These advantages can lead to a simple and rapid process with high recovery. In general, mouse ascites fluids containing mAbs are precipitated with 50% saturated ammonium sulfate (1.9 M). The precipitates are collected and dissolved with the HPLC starting buffer, which contains ammonium sulfate (generally 1 M), and applied to hydrophobic interaction HPLC. After completely washing the column with the same buffer, the ammonium sulfate gradient is applied. By reducing the concentration of ammonium sulfate, $F(ab')_2$ fragments and IgG1 elute separately from the gel at 0.3 M and 0.2 M, respectively. The fraction containing $F(ab')_2$ fragments is homogeneous (Fig. 4), and can be used directly for immunological reactions. If necessary, the remaining ammonium sulfate can be removed by gel filtration or dialysis.

The pepsin digestion time can be greatly reduced by centrifugation (10,000 x g, 20 min) prior to digestion time after ascites fluids are precipitated with 50% saturated ammonium sulfate. It takes 12-48 h for the digestion in the methods previously reported (1, 2), however, the author showed in this section that 2 h are sufficient. The effect of the centrifugation is supposedly that lipids and contaminating proteins which prevent the pepsin action are removed.

It is noteworthy that all IgG1 mAbs elute at almost the same retention times (32-35 min) as well all $F(ab')_2$ fragments (27-30 min) under the conditions shown in Fig. 3. This suggests that the IgG1 and $F(ab')_2$ fragments are almost identical with regard to hydrophobicity. On the other hand, the elution times of IgG1 and $F(ab')_2$ fragments in ion-exchange chromatography are different reflecting their hydrophilicity.

By using the procedures provided in this section, the cycle-time of the hydrophobic interaction HPLC was 45 min, and 9-2200 mg of $F(ab')_2$ fragments were obtained from each cycle. It takes 30 h to purify $F(ab')_2$ from a collection of ascites fluids. The procedure are thought to be suitable for large-scale preparation of $F(ab')_2$ fragments of IgG1 mAbs.

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Section 2

Single-step Purification of F(ab')$_{2\mu}$ Fragments of Mouse Monoclonal Antibodies (Immunoglobulins M) by Hydrophobic Interaction High-performance Liquid Chromatography

Introduction

Immunoglobulin M (IgM) is a common immunoglobulin in sera or secreted from hybridoma cell lines. IgM molecules have a molecular mass of approx. 1,000 kDa. Because of their size, the number of application of IgM is restricted. In particular, in immunohistochemical studies, IgM can not penetrate tissues sufficiently. In enzyme immunoassay, IgM has been generally avoided because of its low solubility and difficulty in handling. Therefore, suitable methods have been desired for producing active fragments of IgM.

Standard methods have been established for cleaving rabbit IgG into active fragments, Fab and F(ab')$_2$. Both pepsin and papain have been used to produce these fragments (1-6). Similarly, methods have been described for producing small fragments of human IgM (7-12). The disulfide-bridge arrangement in the mouse IgM is different from that in human or rabbit IgM (13). Consequently, procedures to generate small fragments from human IgM have not been successful when applied to mouse IgM. In a study by Gorini et al. (14), pepsin digestion of mouse IgM antibodies resulted in three fragments, the largest of which resembled F(ab')$_2$ fragments. Using similar methodology, Beal and Van Dort (15) reported

that they could generate F(ab')$_2$ fragments, as well as Fab fragments. Matthew and Reichardt (16) obtained active fragments of 110 kDa and 230 kDa by trypsin digestion of a mouse IgM. Bidlack and Mabie (17) devised the protocol for the production of Fab fragments of 48 kDa from mouse IgM (18-20). In a separate study, Maillet et al. (21) observed that upon subjecting an IgM mAb to pepsin digestion at 37°C, only a 20% yield of F(ab')$_2$ fragments was obtained. Recently, Pascual and Clem (22) reported a procedure to produce F(ab')$_2$ fragments of 134 kDa in a high yield by pepsin digestion at 4°C, and pH 4.0.

In this section, the author describes a new method for the preparation of F(ab')$_2$ fragments of 144-146 kDa from pepsin digests of mouse IgM mAbs by hydrophobic interaction HPLC using TSKgel Ether-5PW (Fig. 9). The author shows that this method is suitable for large-scale purification of F(ab')$_2$ fragments with a high yield. Hereinafter the author uses the term F(ab')$_{2\mu}$ instead of F(ab')$_2$ fragments of IgG1, according to Johnstone and Thorpe (19) in order to indicate that the fragments are prepared from IgM by cleaving the heavy μ-chains.

Materials and Methods

mAbs

Five mouse mAbs of IgM class were used: TS2M, TS3M, TS4M, TS7M, and TS9M. The specific antigen for TS2M, TS3M and TS4M is human carcinoembryonic antigen (CEA), that for TS7M and TS9M is human myoglobin. The light (L) chain type of all mAbs is κ. The hybridomas secreting these mAbs were established in the laboratory the author

belongs to (unpublished data), by fusing spleen cells from an antigen-immunized BALB/c mouse with SP2/0-Ag.14 mouse myeloma cells according to Köhler and Milstein (24). The hybridoma cells were injected into pristane-primed BALB/c mice, and were grown in ascites fluids (25). mAbs were purified from the ascites fluids at 4°C. The collected ascites fluids were centrifuged at 3,000 x g for 20 min to remove cells and the supernatants were passed through Millipore filters (pore size: 0.8 μm; AA type). Ammonium sulfate (solid) was added to the filtrate to give 60% saturation, followed by centrifugation at 10,000 x g after standing for 2 h. The precipitates were dissolved in phosphate-buffered saline, pH 7.4 (PBS). Ammonium sulfate (solid) was added to give 60% saturation and the precipitates were collected by centrifugation as above, and dissolved in 100 mM citrate buffer (pH 4.2) to give a protein concentration of 0.5-5 mg/ml. Undissolved materials were removed by centrifugation at 10,000 x g for 20 min. The supernatant was adjusted to pH 4.2 with 1 M HCl or NaOH, giving partially purified mAb.

Pepsin digestion

The partially purified mAb was digested by porcine pepsin (EC 3.4.23.1) (Lot No. 117F-8080; 3900 U/mg according to the supplier) purchased from Sigma (St. Louis, MO). The starting concentration of mAb was 0.5-5 mg/ml in 100 mM citrate buffer (pH 4.2). Pepsin was added to the mAb solution at a weight ratio of 1:200 (pepsin : IgM). Digestion proceeded with gentle stirring at 37°C for 2 h and was stopped by adding 10 volumes of 3 M tris(hydroxymethyl)aminomethane (Tris) to give a pH around 7.

High performance liquid chromatography (HPLC)

The HPLC apparatus consisted of a solvent-delivery system CCPM, a UV monitoring

system UV8010, a fraction collector FC8000 and a computer control system SC8010 was purchased from Tosoh (Tokyo). The elution was monitored by absorbance at 280 nm, and each fraction (1 ml) were collected.

Hydrophobic interaction HPLC was performed on a TSKgel Ether-5PW column (7.5 mm (inner diameter) x 75 mm) (Tosoh). Pepsin digests of mAbs were salted out with 60% saturated ammonium sulfate, and the precipitates were immediately dissolved in PBS containing 1 M ammonium sulfate (pH 7.4). The solution was applied to the column equilibrated with the same buffer, and eluted with a linear gradient of ammonium sulfate from 1 to 0 M in PBS (pH 7.4), for 30 min at a flow-rate of 1 ml/min at room temperature.

Gel-filtration HPLC was performed using a TSKgel G4000SW$_{XL}$ column (7.8 mm (inner diameter) x 30 cm) (Tosoh) with PBS (pH 7.4) at a flow-rate of 1 min/min.

Analytical studies

SDS-PAGE was performed in 12% and 6% slab gels under reducing and non-reducing conditions, respectively, according to the method of Laemmli (26). Proteins were reduced by treatment with 2.5% 2-mercaptoethanol at 100°C for 10 min. Proteins were stained with Coomassie brilliant blue R-250. The molecular mass marker kit containing rabbit muscle phosphorylase b (97 kDa), bovine serum albumin (BSA) (66 kDa), chicken ovalbumin (43 kDa), bovine erythrocyte carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and bovine milk α-lactalbumin (14.2 kDa) is a product of Pharmacia (Uppsala, Sweden). Rabbit muscle myosin (200 kDa) and *Escherichia coli* ß-galactosidase (116 kDa) are from Daiichi Chemicals (Tokyo, Japan), and horse plasma α_2-macroglobulin (170 kDa) is from Boehringer Mannheim Yamanouchi (Tokyo, Japan). Isoelectric points

(p*I* values) were determined by isoelectric focusing using carrier ampholyte, Ampholine, for pH 3.5-9.5 (Pharmacia, Uppsala, Sweden).

Concentration of $F(ab')_{2\mu}$ and mAb (IgM) were determined by the method of Lowry et al. (27) with BSA as the standard.

Neutral sugars were estimated by the phenol-sulfuric acid method of Dubois et al. (28).

Binding of $F(ab')_{2\mu}$ and mAb (IgM) to human C1q complements was measured by a solid-phase immunoassay (29). A microtiter plate (96 wells; Nunc-Intermed, MaxiSorp; Roskilde, Denmark) was coated with 100 µl $F(ab')_{2\mu}$ or 100 µl mAb at various concentrations (5, 2.5, 1.2, 0.6, 0.3, and 0 µg/ml) in PBS to each well, followed by incubation for 2 h at 25°C. Plates were blocked by incubation with 0.2% BSA in PBS overnight at 4°C. The plate was washed with PBS and incubated with 20% human serum in PBS for 2 h at 25°C. After washing the plate once more with PBS, excess goat anti-human C1q antibody conjugated with horseradish peroxidase (The Binding Site, Birmingham, UK) was added to the plate and incubated for 2 h at 25°C. The plate was washed with PBS, then the enzyme reaction was started by adding 100 µl substrate (0.03% ABTS and 0.03% H_2O_2 in 100 mM citrate buffer, pH 4.1) to each well, and then terminated by 100 µl oxalic acid (0.1 M) after reaction for 5 min at 25°C. The absorbance at 415 nm was measured by a microtiter plate reader MPR-A4 (Tosoh).

Antigen-binding activities of $F(ab')_{2\mu}$ and mAb (IgM) were measured by a solid-phase enzyme immunoassay (sandwich assay) (30). A 96-wells microtiter plate was coated with 100 µl of $F(ab')_{2\mu}$ or mAb at 2 µg/ml in PBS to each well, followed by incubation for 2 h at 25°C. Plates were blocked by incubation with 0.2% BSA in PBS overnight at 4°C. The plate was washed with PBS, and incubated with 50 µl of antigen at various concentrations (1.0, 0.5, 0.25, 0.12, 0.06, 0.03, and 0 ng/ml) in PBS and 100 µl of mouse anti-antigen

monoclonal antibody (IgG1) conjugated with horseradish peroxidase for 2 h at 25°C. Then, the plate was washed with PBS, and the enzyme reaction was done as described above. The IgG1 mAbs used here were all prepared in our laboratory.

Results

Pepsin digestion

Time-dependence of pepsin digestion of IgM (TS2M; mouse anti-human CEA mAb) was monitored by SDS-PAGE (Fig. 1). After the incubation time indicated in Fig. 1, the reaction was stopped and the reaction mixture was applied to SDS-PAGE (12% gel) under reducing conditions. IgM shows two bands before the reaction, of 75 kDa and 27 kDa, which correspond to heavy (H) and light (L) chains, respectively. The H-chain disappeared in the reaction, and a new band of 47 kDa appeared . The L-chain did not appear to be degraded in the reaction. After a 2-h digestion, the H-chain band disappeared completely, and only 47-kDa and 27-kDa bands remained. The new 47-kDa band must be derived from cleavage of the H (µ) chain. Similar time-courses of pepsin digestion were obtained for four other mAbs (IgM) examined (data not shown).

Purification of $F(ab')_{2\mu}$ fragments by hydrophobic interaction HPLC

The pepsin digests were separated by hydrophobic interaction HPLC using a TSKgel Ether-5PW column. Chromatograms for the pepsin digests of mAb TS2M obtained by the digestion for 0, 30, and 120 min are shown in Fig. 2. A linear gradient of ammonium sulfate from 2.0 to 0 M was generated over 30 min, and the elution was continued using

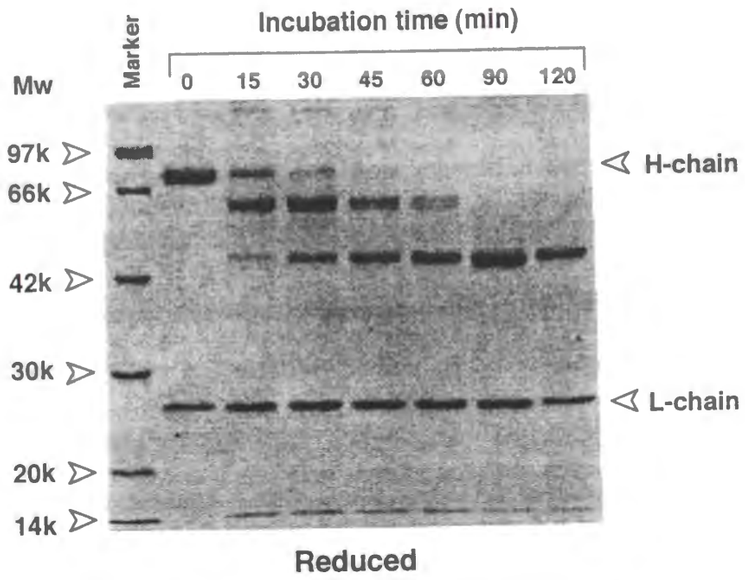

Fig. 1. SDS-PAGE of mouse mAb TS2M (IgM) digested with pepsin at the pepsin : IgM ratio of 1 : 200 (w/w) at 37°C in 100 mM citrate buffer (pH 4.2) for various incubation times indicated. SDS-PAGE was done under reducing conditions. Prior to SDS-PAGE, the digests were treated with 2.5% 2-mercaptoethanol at 100°C for 10 min.

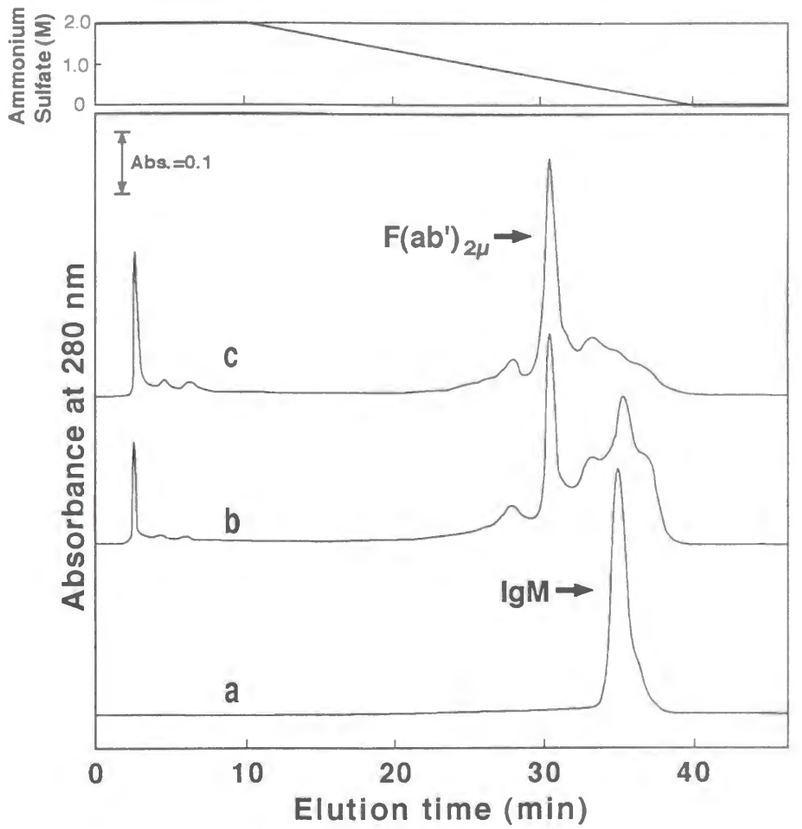

Fig. 2. Purification of mouse $F(ab')_{2\mu}$ fragments from pepsin digests of mAb TS2M by hydrophobic interaction HPLC on a TSKgel Ether-5PW column. Incubation time with pepsin: a, 0 min; b, 30 min; c, 120 min.

ammonium-sulfate-free PBS. The mAb eluted at 36.0 min, and as the pepsin digestion progressed, the height of the mAb peak decreased. The peak disappeared completely after digestion for 120 min, and inversely, a new peak corresponding to $F(ab')_{2\mu}$ fragments appeared at 31 min. Fractions from 30 to 32 min were collected for further analysis.

Purity of $F(ab')_{2\mu}$ fragments

The fractions collected from 30 to 32 min in the hydrophobic interaction HPLC (Fig. 2) were applied to gel-filtration HPLC on a TSKgel G4000SW$_{XL}$ column (Fig. 3). The proteins eluted as a single peak at 11.0 min, considerably behind the retention time of IgM TS2M (7.8 min), showing that the purity of the $F(ab')_{2\mu}$ fragments was more than 98%. SDS-PAGE of the fractions under non-reducing conditions (Fig. 4A) showed a single band of 144-146 kDa, and SDS-PAGE under reducing conditions (Fig. 4B) showed only two bands corresponding to H (47 kDa) and L (27-29 kDa) chains. Therefore, $F(ab')_{2\mu}$ was considered to be composed of two sets of H- and L-chains. Contaminating proteins observed before the hydrophobic interaction HPLC (Fig. 1) were removed. The mobility of the L-chain of $F(ab')_{2\mu}$ fragments from TS2M, TS7M, and TS9M is slightly different, although it is almost the same as that of L (κ) chain of original mAbs. In Fig. 4B, SDS-PAGE of only TS9M was shown. The purification processes are summarized in Table 1. In the case of mAb TS2M, the total protein of 28 mg was applied to pepsin digestion. When the total mAb was theoretically and entirely converted to $F(ab')_{2\mu}$ by pepsin digestion, namely, one IgM molecule of 900 kDa is converted to five $F(ab')_{2\mu}$ molecules of 144 kDa, the ratio of $F(ab')_{2\mu}$ should be 80% (i. e., (144x5)/900=0.8) of the mAb quantity, namely 22 mg. The quantity of the latter obtained from the hydrophobic interaction HPLC was 12 mg, thus, the yield of $F(ab')_{2\mu}$ was 55%. The material balances of other mAbs are listed in

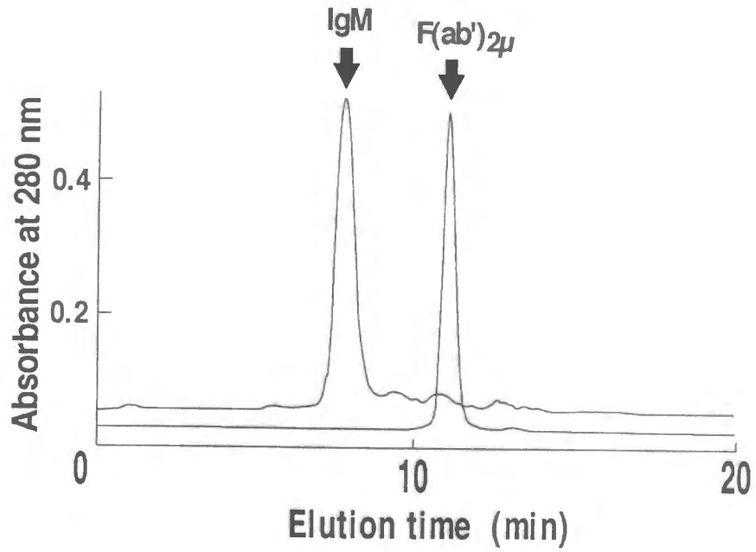

Fig. 3. Analytical gel-filtration HPLC of $F(ab')_{2\mu}$ fragments purified from pepsin-digests of mAb TS2M by hydrophobic interaction HPLC on a TSKgel Ether-5PW column. The gel-filtration HPLC was done on a TSKgel G4000SW$_{XL}$ column.

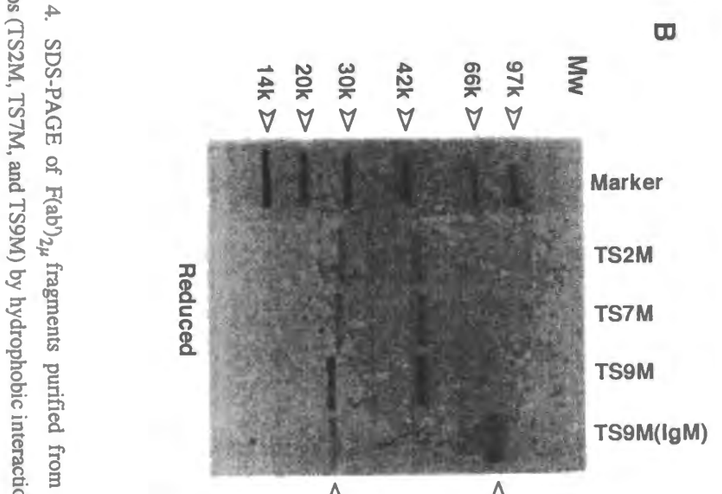

Fig. 4. SDS-PAGE of $F(ab')_{2\mu}$ fragments purified from pepsin-digests of IgM mAbs (TS2M, TS7M, and TS9M) by hydrophobic interaction HPLC on a TSKgel Ether-5PW column (Fig. 2). A: non-reducing conditions; the fragments were applied to SDS-PAGE without 2-mercaptoethanol treatment. B: reducing conditions; prior to SDS-PAGE, the fragments were treated with 2.5% 2-mercaptoethanol at 100°C for 10 min. In the lane indicated by TS9M (IgM), mAb TS9M was subjected to SDS-PAGE as the control.

Table 2

Sugar contents of mouse IgM monoclonal antibodies and their $F(ab')_{2\mu}$ fragments

Monoclonal antibody	Sugar content [a] (%, w/w)		Ratio of sugar contents of $F(ab')_{2\mu}$ to IgM
	IgM	$F(ab')_{2\mu}$	
TS2M	8.8	3.9	0.44
TS3M	11.0	3.7	0.34
TS4M	10.0	3.5	0.35
TS7M	5.8	4.2	0.72
TS9M	6.6	4.1	0.62

[a] The quantity of neutral sugar was estimated by the phenol-sulfuric acid method of Dubois et al.. The sugar content (%, w/w) was obtained as ((Quantity of sugar (mg)) / (Quantity of protein including sugar (mg))) x 100.

Table 1

Purification of mouse F(ab')$_{2\mu}$ fragments by hydrophobic interaction HPLC by TSKgel Ether-5PW and their molecular masses and isoelectric points

Monoclonal antibody	IgM used for pepsin digestion [a] (mg)	F(ab')$_{2\mu}$ purified [b] (mg)	Recovery yield of F(ab')$_{2\mu}$ [c]	Purity (%)	Isoelectric point of F(ab')$_{2\mu}$	Molecular mass of F(ab')$_{2\mu}$ (kDa)
TS2M	**28**	**12**	**0.55**	**98**	**6.0**	**144**
TS3M	**54**	**31**	**0.72**	**98**	**6.0**	**144**
TS4M	**220**	**98**	**0.56**	**97**	**6.3**	**144**
TS7M	**70**	**32**	**0.57**	**98**	**7.0**	**144**
TS9M	**6**	**3**	**0.62**	**97**	**6.7**	**146**

[a] Quantity of IgM obtained after precipitation of ascites with 60% saturated ammonium sulfate.

[b] Quantity of F(ab')$_{2\mu}$ fragments purified by hydrophobic interaction HPLC using TSKgel Ether-5PW.

[c] (Quantity of F(ab')$_{2\mu}$ (mg)) / (Quantity of IgM (mg) x 0.8).

Table 1. The purity of F(ab')$_{2\mu}$ was greater than 97% and the recovery yield was in the range of 55-72%. *p*I values of F(ab')$_{2\mu}$ fragments are also listed in Table 1. They are in the range of 6.0-7.0. On the other hand, those of mAbs could not be measured because their molecular size was too large to migrate in the carrier ampholine. Sugar contents of F(ab')$_{2\mu}$ fragments and mAbs are listed in Table 2. The sugar contents of mAbs were in the range of 5.8-11.0% (31), and those of F(ab')$_{2\mu}$ were between 3.5 and 4.2%. In every case, the sugar content was decreased by fragmentation of mAb to F(ab')$_{2\mu}$, however, the degree of the decrease is considerably variable, from 34% (TS3M) to 72% (TS7M).

Fig. 5 shows the binding activities of mAbs and their F(ab')$_{2\mu}$ fragments to human C1q complements. All mAbs showed the C1q binding. On the other hand, F(ab')$_{2\mu}$ fragments lacked the activity completely.

Fig. 6 shows immunoreactivities of mAbs (TS2M and TS9M) and their F(ab')$_{2\mu}$ fragments against their specific antigens, human CEA and myoglobin, respectively. The horseradish peroxidase activity observed by absorbance at 415 nm, which corresponds to the immunoreactivity, increased with the increase of the antigen concentration almost linearly in the range of antigen concentrations used. The slopes of mAb and F(ab')$_{2\mu}$ were almost the same for the both antigens. The absorbance observed at the antigen concentration of zero is due to the non-specific binding of the peroxidase-labeled antibody to the plate and/or antibody coated onto plate. In the case of CEA binding (Fig. 6A), absorbance at 415 nm of the non-specific binding for mAb (TS2M) was 0.075 and that for the F(ab')$_{2\mu}$ was 0.015. In the case of myoglobin (Fig. 6B), the values of mAb (TS9M) and the F(ab')$_{2\mu}$ were 0.19 and 0.01, respectively. In both cases, the non-specific binding was reduced greatly by using F(ab')$_{2\mu}$ fragments in place of IgM mAbs.

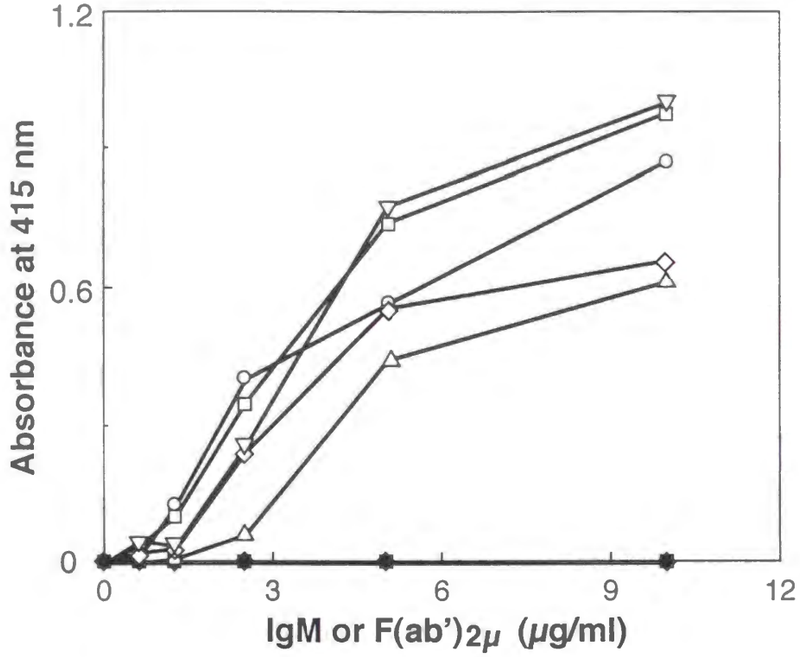

Fig. 5. The binding activities of IgM mAbs and $F(ab')_{2\mu}$ fragments to human C1q complements. Each well of a 96-well microtiter plate was coated with $F(ab')_{2\mu}$ fragments or IgM mAbs by adding their solution of 100 μl at the concentrations shown in the horizontal axis. The plate was incubated with horseradish peroxidase. Absorbance at 415 nm generated by the reaction with ABTS for 5 min was observed. IgM and $F(ab')_{2\mu}$, respectively: ○ and ● for TS2M; □ and ■ for TS3M; △ and ▲ for TS4M; ◇ and ◆ for TS7M; ▽ and ▼ for TS9M.

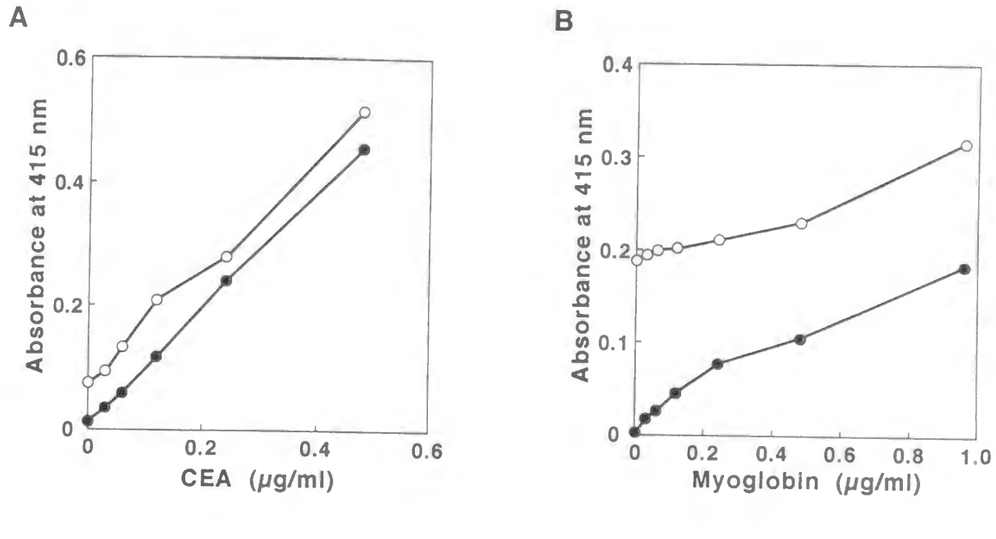

Fig. 6. Immunoreactivities of mAbs (TS2M and TS9M) and their $F(ab')_{2\mu}$ fragments against their specific antigens, CEA and myoglobin, respectivity: IgM (○) or $F(ab')_{2\mu}$ (●). Each well of a 96-well microtiter plate was coated with antigen by adding 100 μl antigen solution at the concentrations shown in the horizontal axis. 100 μl of 2 μg/ml mAb or $F(ab')_{2\mu}$ was added, followed by mouse mAb (IgG1), specific to the antigen, conjugated with peroxidase. Absorbance at 415 nm generated by the reaction with ABTS for 5 min was observed. A, TS2M; B, TS9M.

Discussion

$F(ab')_{2\mu}$ fragments of mouse mAbs are currently of great interest for both diagnostic and therapeutic agents (20, 32). They are more useful than the original mAbs, because they do not retain any biological function due to Fc regions and their interaction with non-specific proteins is reduced. A smaller molecular mass than the mAbs is another advantage. From this point of view, methods for preparation of $F(ab')_2$ and Fab fragments from IgG mAbs have been examined (1-6). On the other hand, the method for fragmentation of IgM mAbs is not yet well fixed. In this section, the author intended to establish a method for the preparation of $F(ab')_{2\mu}$ fragments from mouse IgM mAbs with a single-step chromatographic procedure.

IgM is much more difficult to handle than IgG because of its large molecular size. Consequently, IgM-producing hybridomas have been disliked generally in selection of the cell lines. However, most of the hybridomas raised against carbohydrate antigens, including tumor-associated antigens, are somehow IgM producers, and efforts have been paid to select class-switch variants from hybridoma clones producing IgM (33, 34). The $F(ab')_{2\mu}$ fragments of mouse mAbs of the IgM isotype described here could make them more applicable to immunoassay and immunohistochemistry.

The author described the application of hydrophobic interaction HPLC using TSKgel Ether-5PW to the single-step purification of $F(ab')_{2\mu}$ fragments of mouse mAbs of the IgM isotype. The procedure was designed basically to follow the method successfully applied in the purification of $F(ab')_{2\mu}$ fragments of mouse IgG1 mAbs (6). One of the features of this HPLC is that ammonium sulfate can be used as the salt for controlling the adsorption

and removal of the proteins. Ammonium sulfate is widely used as a salting-out reagent in protein purification, because its effects on proteins are mild and the solubility is considerably high. In our experience, mAbs (IgM) and $F(ab')_{2\mu}$ fragments are stable in the presence of ammonium sulfate. In general, mouse ascites containing IgM mAbs are precipitated with 60% saturated ammonium sulfate (2.5 M). The precipitates are collected and dissolved with the HPLC starting buffer, which contains ammonium sulfate (generally 1 M), and applied to hydrophobic interaction HPLC. After completely washing the column with the same buffer, the ammonium sulfate gradient is applied. By reducing the concentration of ammonium sulfate, $F(ab')_{2\mu}$ fragments and mAbs elute separately from the gel at 0.28 M and 0.17 M, respectively. $F(ab')_{2\mu}$ fragments are less hydrophobic than the IgM mAbs, similar to that $F(ab')_2$ fragments of IgG1 are less hydrophobic than their original mAbs (6). The fraction containing $F(ab')_{2\mu}$ fragments is homogeneous (Fig. 3 and 4), and can be used directly for immunological reactions. If necessary, the remaining ammonium sulfate can be removed by gel-filtration or dialysis.

The advantages of the purification of $F(ab')_{2\mu}$ fragments described here are not only that the procedure takes only a single step, but also that no buffer exchange, such as dialysis, is required. These advantages can lead to a simple and rapid process with high recovery. The cycle time of the hydrophobic interaction HPLC was 40 min, and 3-98 mg of $F(ab')_{2\mu}$ fragments were obtained from each cycle. It takes 30 h to purity $F(ab')_{2\mu}$ from a collection of ascites. The procedures provided here are thought to be suitable for large-scale preparation of $F(ab')_{2\mu}$ fragments of IgM mAbs.

Another gel for hydrophobic interaction HPLC, TSKgel Phenyl-5PW was also tested in the separation of $F(ab')_{2\mu}$ fragments from IgM mAbs under the same conditions as in Fig. 2. This gel was well applicable (data not shown), however, the elution times of $F(ab')_{2\mu}$ and

mAb were 33 min and 37 min, respectively, which is longer than those obtained by TSKgel Ether-5PW (Fig. 2), reflecting difference in hydrophobicity between both gels.

Although so far there have been some trials in fragmentation of IgM (7-12, 14-17, 21, 22), the proteolytic conditions applied were various, and the products were diverse, and even if the products were referred to $F(ab')_2$ fragments, their molecular sizes were rather different. Recently, Pascual and Clem (22) described that pepsin digestion at low temperature (4°C) produced the $F(ab')_2$ fragments (134 kDa) of mouse IgM effectively when compared to production at 37°C. They carried out the digestion for 24 h at a considerably high ratio (w/w) of pepsin to mAb (1:25 at 4°C; 1:100 at 37°C) at pH 4.0. In this section, the author showed that pepsin digestion at 37°C (pH 4.2) at the pepsin-to mAb ratio of 1:200 (w/w) for 2 h gave a homogeneous fraction of $F(ab')_{2\mu}$ fragments in high yield (55-72%). The molecular mass of $F(ab')_{2\mu}$ fragments in our method is 144-146 kDa, which is almost 10 kDa higher than that of the fragments of Pascual and Clem (22). As light chains remained to be intact in both methods, the cleaved position in the heavy chains may be different, depending on the conditions of pepsin digestion.

On our experience, the pepsin digestion time can be greatly reduced by centrifugation (10,000 x g, 20 min) prior to digestion after ascitic fluids are precipitated with 60% saturated ammonium sulfate. It takes 4-24 h for the digestion in the methods previously reported (9, 19, 22), however, the author showed here that 2 h are sufficient. The same effect was observed in the fragmentation of mouse IgG1 mAbs by pepsin digestion (6). The effect of the centrifugation is supposedly that lipids and contaminating proteins which prevent the pepsin action are removed.

By using $F(ab')_{2\mu}$ fragments instead of IgM mAbs in enzyme immunoassay, non-specific binding was reduced to a great extent (Fig. 6), suggesting that accuracy of the assay could

be improved. Non-specific binding, as well as complement C1q binding of IgM mAbs, may be plausibly due to sugar moieties in the Fc regions which are split off by the pepsin digestion (Table 2) (35).

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Section 3

Preparation of F(ab')$_{2\mu}$ Fragments from Rat IgM Monoclonal Antibodies and their Application to the Enzyme Immunoassay of Mouse Interleukin-6

Introduction

Interleukin-6 (IL-6) is one of important molecules in the immune response, the acute phase reaction, the induction of fever and hematopoiesis, and that its abnormal expression is linked to various diseases (1-3).

The measurement of IL-6 in the blood and urine of not only human but also the experimental animals is considered to be important (4, 5). Hell et al. reported that they could develop a sensitive ELISA for human IL-6 (6). Recently, although sandwich enzyme immunoassay for murine IL-6 was established by rat IgM monoclonal antibodies (7), the sensitivity and reproducibility were not satisfied.

The immunoglobulin M (IgM) molecule is a common and primitive immunoglobulin. As IgM have a large molecular mass, approximately one million, the application of IgM have been restricted. There are some reports in the literature on preparation of mouse F(ab')$_2$ fragments of IgG (8, 9) and mouse F(ab')$_{2\mu}$ of IgM with pepsin (10, 11), though rat IgM has not been reported. The author succeeded the preparation of F(ab')$_{2\mu}$ from rat IgM by pepsin and purification using hydrophobic interaction HPLC. Optimized pepsin digestion led to the recovery yield of up to 41%. The author shows that F(ab')$_{2\mu}$ for sandwich enzyme immunoassay of mIL-6 could be drastically reduced non-specific binding and

improved reproducibility. In this section, the author describes the applicable preparation and purification of rat F(ab')$_{2\mu}$ fragments, and sandwich enzyme immunoassay of mIL-6 using F(ab')$_{2\mu}$ fragments.

Materials and methods

Monoclonal antibodies

Four different rat IgM against mIL-6 were CY-02, CY-04, CY-06, and CY-12. These hybridoma were obtained by fusing spleen cells from a wister rat with YB2/0 rat myeloma cells in our laboratory according to Köhler and Milstein (12). These rat monoclonal IgM used throughout this study were partially purified from ascites fluid by salting out with 60% saturation ammonium sulfate. After centrifugation at 10,000 x g, the precipitate were resuspended in 100 mM citrate buffer (pH 4.5) to give protein concentration of 1-2 mg/ml. The solution were centrifuged at 10,000 x g for 20 min and precipitate was carefully discarded. The supernatant was adjusted to a final pH 4.5 with 1 N HCl or NaOH.

Pepsin digestion

The partially purified IgM was digested with porcine pepsin (EC 3.4.23.1) (3900 U/mg, Sigma, St. Louis, MO) in 100 mM citrate buffer pH 4.5, was added at a weight ratio of 200:1 (IgM:pepsin, w/w). The sample was incubated at 37°C for 2 h. The digestion was terminated to raise neutral pH by the addition of 3 M Tris at 1/10 (v/v) of the solution.

High-performance liquid chromatography (HPLC)

Hydrophobic interaction HPLC was performed on a TSKgel Phenyl-5PW column (7.5 mm (inner diameter) x 7.5 cm) (Tosoh). The products of pepsin digestion were salted out with 50% saturated ammonium sulfate, and the precipitates were dissolved with PBS containing 1 M ammonium sulfate pH 7.4 (starting buffer), immediately. The sample were loaded on the column in starting buffer and eluted with a linear gradient to PBS, for 30 min.

Gel-filtration HPLC was performed using a TSKgel G3000SW$_{XL}$ column (7.8 mm (inner diameter) x 30 cm) (Tosoh) with PBS at a flow rate of 1.0 ml/min.

Analytical studies

SDS-PAGE was carried out in a 12% slab gel under reducing conditions and 6% slab gel under non-reducing conditions according to the method of Laemmli (13). Gels were stained with Coomassie blue R-250. The molecular weight markers consisted of rabbit muscle myosin (200 kDa), *Escherichia coli* β-galactosidase (116 kDa), rabbit muscle phosphorylase b (97 kDa), bovine serum albumin (66 kDa), rabbit muscle aldolase (42 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa), and lysozyme (14 kDa).

Protein concentrations were determined according to the optical density at 280 nm. The extinction coefficient were used 1.2 for 1% solution.

Neutral sugars of IgM mAbs and their F(ab')$_{2\mu}$ were estimated by the phenol-sulfuric acid method.

Binding of F(ab')$_{2\mu}$ and mAb (IgM) to human C1q complements was measured by a solid-phase immunoassay. A microtiter plate (96 wells; Nunc-Intermed, MaxiSorp; Roskilde, Denmark) was coated with 100 µl F(ab')$_{2\mu}$ or 100 µl mAb at various concentrations (5, 2.5, 1.2, 0.6, and 0 µg/ml) in PBS to each well, followed by incubation

for 2 h at 25°C. Plates were blocked by incubation with 0.2% BSA in PBS overnight at 4°C. The plate was washed with PBS and incubated with 20% human serum in PBS for 2 h at 25°C. After washing the plate once more with PBS, excess goat anti-human C1q antibody conjugated with horseradish peroxidase (The Binding Site, Birmingham, UK) was added to the plate and incubated for 2 h at 25°C. The plate was washed with PBS, then the enzyme reaction was started by adding 100 µl substrate (0.03% ABTS and 0.03% H_2O_2 in 100 mM citrate buffer, pH 4.1) to each well, and then terminated by 100 µl oxalic acid (0.1 M) after reaction for 5 min at 25°C. The absorbance at 415 nm was measured by a microtiter plate reader MPR-A4 (Tosoh).

The immunoreactivity of two IgM mAbs (CY-04 and CY-06) and their F(ab')$_{2\mu}$ fragments were measured by means of a solid-phase sandwich enzyme immunoassay (14). Microtiter plates (Nunc, MaxiSorp) were coated with anti-murine IL-6 mAb CY-04, CY-06 IgM or their F(ab')$_{2\mu}$ in 50 mM carbonate buffer (pH 9.5) of 2 µg/ml at 37°C for 1 h and blocked by 0.2% BSA in PBS. The plates were washed with PBS, and incubated with 100 µl of mIL-6 at various concentrations and 100 µl of guinea pig anti-murine IL-6 polyclonal antibody (3 µg/ml; prepared according to Saito et al. (7)) for 2 h at 25°C. Washed plates were incubated with alkaline phosphatase conjugated rabbit anti-guinea pig IgG (BioMakor, Rehovot, Israel) at 37°C for 1 h. Finally, the plates washed were incubated with substrate (1.0 mg/ml pNPP in 50 mM carbonate, pH 9.5 containing 10 mM $MgCl_2$) and measured absorbance at 405 nm.

Results

Pepsin digestion

Pepsin digests of IgM, CY-04, prepared as described above, were monitored by SDS-PAGE (Fig. 1). After the indicated incubation periods, the reaction was stopped and the mixture was resolved by SDS-PAGE under reducing conditions. IgM peptides migrated as 75 and 27 kDa bands corresponding to heavy (H) and light (L) chains, respectively before the reaction. The H chain disappeared during the reaction, and two new bands of 44 and 48 kDa appeared. These bands appeared very early in the reaction, and maintained mutually the same density regardless of the reaction period. After a 2 h digestion, the H chain band disappeared completely, and only 44, 48, and 27 kDa bands remained. The L chain seemed not to be degraded in the reaction. The first two, as well as the 75 kDa band were confirmed to be derived from cleavage of the H (μ) chain (data not shown). Similar time courses of pepsin digestion were obtained for three other mAbs (IgM) examined (data not shown).

Purification of $F(ab')_{2\,\mu}$ fragments by Hydrophobic interaction HPLC

The sample which were subjected to hydrophobic interaction HPLC using a TSKgel Phenyl-5PW column. Fig. 2 shows the chromatograms of mAb CY-04 and the pepsin digests. In this condition, the mAb eluted at 43.2 min. As the pepsin digestion progressed, the peak height of the mAb decreased and disappeared completely after digestion for 2 h. $F(ab')_{2\mu}$ fragments appeared at 34.2 min and fractions from 33.5 to 35.2 min were collected for further analysis. Other $F(ab')_{2\mu}$ were also separated from IgMs with hydrophobic interaction HPLC (data not shown) as well as CY-04. The resulting CY-04 $F(ab')_{2\mu}$ was subjected to gel filtration HPLC on a TSKgel G3000SW$_{XL}$ column (Fig. 3). The original IgM mAb eluted at 5.4 min. The proteins eluted as a single peak at 9.0 min showing that

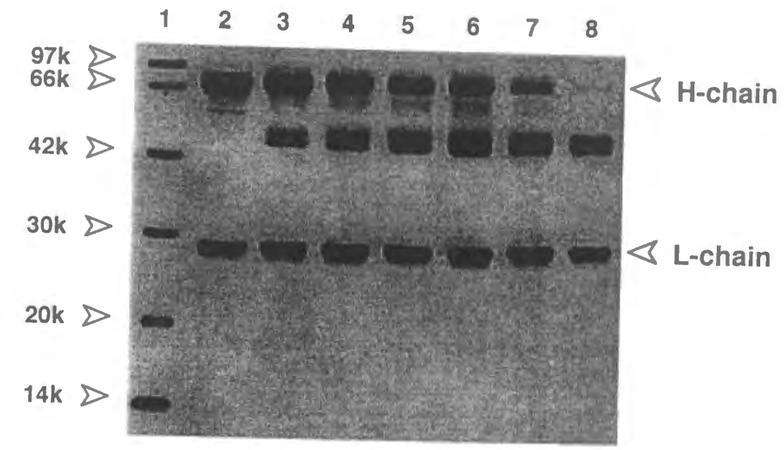

Fig. 1. SDS-PAGE of rat mAb CY-04 (IgM) digested with pepsin at the pepsin:mAb ratio of 1:200 (w/w) at 37°C in 100 mM citrate buffer (pH 4.5) for the various incubation periods indicated. SDS-PAGE proceeded under reducing conditions. Prior to SDS-PAGE, the digests were boiled with 2.5% 2-mercaptoethanol at 100°C for 10 min. Lane 1, molecular mass marker proteins; Lane 2-8, pepsin digests obtained by digestion for 0, 15, 30, 45, 60, 90, and 120 min, respectively.

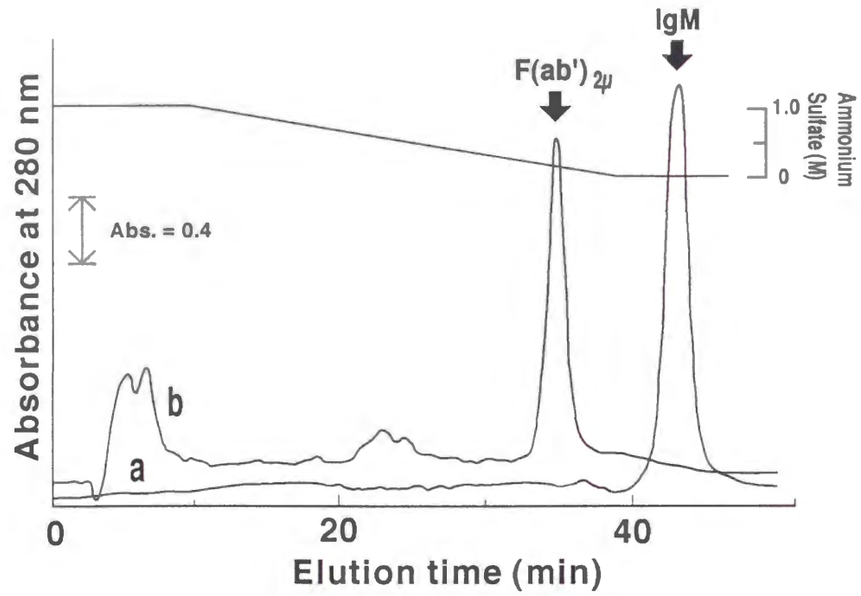

Fig. 2. The purification of rat F(ab')$_{2\mu}$ fragments from pepsin digests of mAb CY-04 by hydrophobic interaction HPLC on a TSKgel Phenyl-5PW column. Incubation time with pepsin: a, 0 min, and b, 120 min.

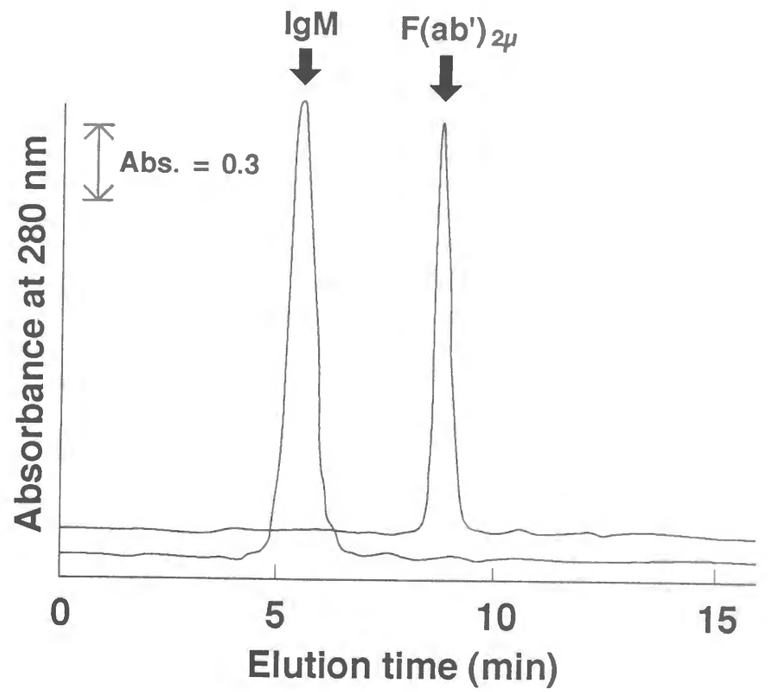

Fig. 3. Analytical gel filtration HPLC of F(ab')$_{2\mu}$ fragments purified from pepsin-digests of mAb CY-04 by hydrophobic interaction HPLC. Gel-filtration HPLC was performed on a TSKgel G3000SW$_{XL}$ column.

the purity of the fragments was above 94%.

Purification of F(ab')$_{2\mu}$

F(ab')$_{2\mu}$ purified with TSKgel Phenyl-5PW chromatography are summarized in Table 1. The recovery yield was in the range of 41-52%. All of the resulting F(ab')$_{2\mu}$ were more than 94% purity by gel filtration HPLC. SDS-PAGE of the fractions under non-reducing conditions (Fig. 4A) showed a single band of 147-155 kDa, and under reducing conditions (Fig. 4B) showed two bands corresponding to H-chain and one band of L-chain. All mAbs were shown the same results in Fig. 4. Therefore, rat F(ab')$_{2\mu}$ was considered to be composed of two set of different H- and L-chains.

The material balance of rat mAbs fragments were listed in Table 1. Sugar contents of rat F(ab')$_{2\mu}$ fragments and mAbs were also listed in Table 1. The sugar contents of mAbs were in the range of 68-140 μg/mg, and those of F(ab')$_{2\mu}$ were between 56-72 μg/mg. In every case, the sugar content was decreased by fragmentation of mAb to F(ab')$_{2\mu}$.

Fig. 5 shows the binding activities of mAbs and their F(ab')$_{2\mu}$ fragments to human C1q complements. All mAbs showed the C1q binding. On the other hand, F(ab')$_{2\mu}$ fragments lacked the activity completely.

Sandwich enzyme immunoassay of mIL-6

Fig. 6 shows the immunoreactivity of two mAbs (CY-04 and CY-06) and their F(ab')$_{2\mu}$ fragments against their specific antigen, mIL-6, as measured by a sandwich enzyme immunoassay. Colour development increased with increasing antigen concentrations for both the intact mAbs and its F(ab')$_{2\mu}$ fragments. Absorbance values observed at the antigen concentration being zero, are due to the non-specific binding of the enzyme-

Table 1

Purification of rat F(ab')$_{2\mu}$ fragments by hydrophobic interaction HPLC by TSKgel Ether-5PW

Monoclonal antibody	IgM used for pepsin digestion [a] (mg)	F(ab')$_{2\mu}$ purified [b] (mg)	Recovery yield of F(ab')$_{2\mu}$ [c]	Purity (%)	Molecular mass of F(ab')$_{2\mu}$ (kDa)	Sugar/Protein [d]	
						IgM (μg/mg)	F(ab')$_{2\mu}$
CY-02	5.3	2.7	0.51	96	147	68	65
CY-04	3.6	1.9	0.52	98	150	140	72
CY-05	3.9	1.6	0.41	100	152	89	61
CY-06	1.3	0.6	0.45	94	153	100	66
CY-12	3.1	1.4	0.46	100	150	87	56

[a] Quantity of IgM obtained after precipitation of ascites with 60% saturated ammonium sulfate.

[b] Quantity of F(ab')$_{2\mu}$ fragments purified by hydrophobic interaction HPLC using TSKgel Phenyl-5PW.

[c] (Quantity of F(ab')$_{2\mu}$ (mg)) / (Quantity of IgM (mg)).

[d] The quantity of neutral sugar was estimated by the phenol-sulfuric acid method. Protein concentrations were determined according to the optical density at 280 nm.

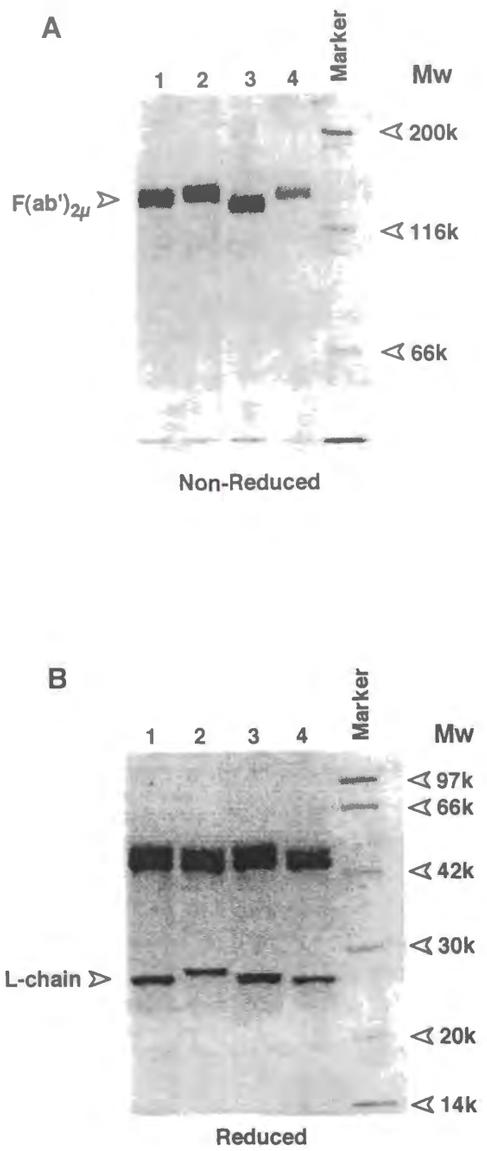

Fig. 4. SDS-PAGE of $F(ab')_{2\mu}$ fragments purified from pepsin-digests of rat IgM mAbs by hydrophobic interaction HPLC (Fig. 2). A: non-reducing conditions; thefragments were applied to SDS-PAGE without mercaptoethanol treatment. B: reducing conditions; prior to SDS-PAGE, the fragments were boiled with 2.5% 2-mercaptoethanol at 100°C for 10 min. Lane 1-4, $F(ab')_{2\mu}$ fragments of rat IgM mAbs CY-02, CY-04, CY-06, and CY-12, respectively.

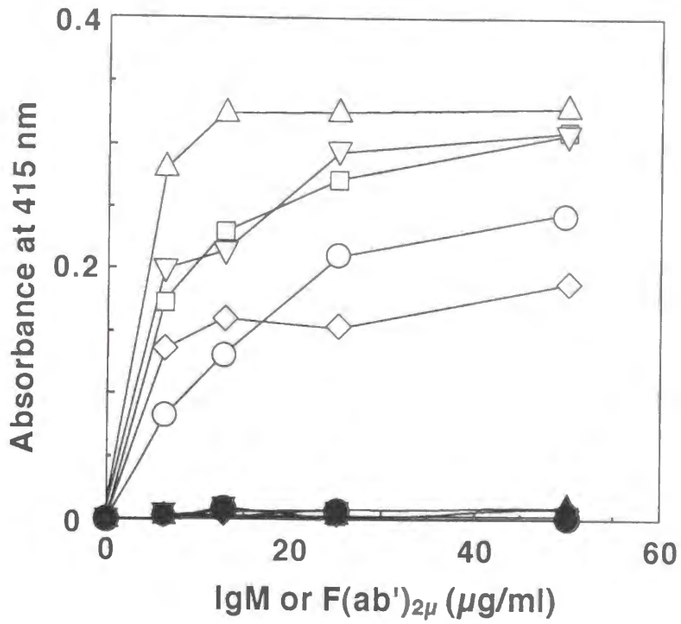

Fig. 5. The binding activities of rat IgM and $F(ab')_{2\mu}$ fragments to human C1q complements. Absorbance at 415 nm generated by the reaction with ABTS for 5 min was observed. IgM and $F(ab')_{2\mu}$, respectively: ○ and ● for CY-02; △ and ▲ for CY-04; ▽ and ▼ for CY-05; □ and ■ for CY-06; ◇ and ◆ for CY-12.

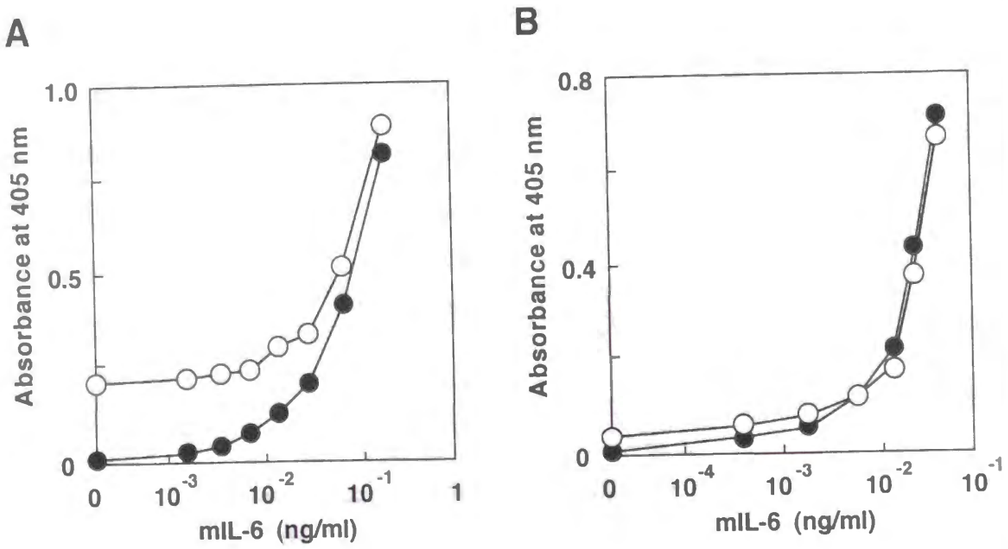

Fig. 6. The immunoreactivities of mAbs (CY-04 and CY-06) and their $F(ab')_{2\mu}$ fragments with mIL-6. The wells of the microtiter plate were coated with 2 μg/ml of mAb (○) or $F(ab')_{2\mu}$ (●) in 100 μl, then each was incubated with 100 μl of mIL-6 at the concentrations indicated on the abscissa. Guinea pig mIL-6 specific antibody was added to the wells, followed by the addition of alkaline phosphatase conjugated with rabbit antibody specific to guinea pig IgG. The absorbance at 405 nm, generated by reaction with pNPP for 10 min, was measured. A, CY-04; B, CY-06. The mIL-6 concentrations in A were 0, 2, 4, 8, 16, 31, 62, and 125 pg/ml, and those in B were 0, 0.4, 2, 6, 12, 24, and 49 pg/ml.

labeled antibody to the plate and/or to the coating antibody (mAb or $F(ab')_{2\mu}$). The absorbance at 405 nm of the non-specific binding for mAb CY-04 was 0.212, and that for $F(ab')_{2\mu}$ was 0.019 (Fig. 6A). The values for mAb CY-06 and its $F(ab')_{2\mu}$ were 0.051 and 0.019, respectively (Fig. 6B). In both cases, the non-specific binding was greatly reduced by replacing the IgM mAbs with the $F(ab')_{2\mu}$ fragments, and the detection limit was determined to be 1 pg/ml by setting a cut-off at the level of the mean plus three standard deviation obtained with zero-dose samples.

Discussion

The author has succeeded to prepare and purify rat $F(ab')_{2\mu}$ fragments from rat IgM mAbs by pepsin digestion. In this section, the author also described the application of rat $F(ab')_{2\mu}$ fragments of IgM mAbs against murine IL-6.

Rat IgM was efficiently digested at a pepsin-IgM ratio of 1:200 (w/w) in 100 mM citrate buffer, pH 4.5 at 37°C for 2 h (Fig. 1). Hydrophobic interaction HPLC using Phenyl-5PW is simple, rapid, and more suitable method for the single-step purification of $F(ab')_{2\mu}$ (Fig. 2). $F(ab')_{2\mu}$ produced by this procedure was more than 94% pure as assessed by gel filtration HPLC (Fig. 3). The recovery yields of $F(ab')_{2\mu}$ were found to be than 41% (Table 1). This methods allows easier and faster preparation of $F(ab')_{2\mu}$. Under reducing conditions, the digested heavy (μ) chain migrated with an apparent molecular weight of 44 and 48 kDa on SDS-PAGE, while the light chain migrated with an apparent molecular weight of 27-28 kDa (Fig. 4). The 44-48 kDa doublets may represent different cleavage points in the μ chain or possibly two populations differing in their carbohydrate composition. However,

under non reducing conditions, the $F(ab')_{2\mu}$ shown one band of 147-152 kDa. Murine IgM are efficiently digested by pepsin at pH 4.2 (11), and the resulting 145 kDa $F(ab')_{2\mu}$ fragments consist of truncated H and L chains of 47 and 28 kDa, respectively. Rat IgM mAbs were also degraded into $F(ab')_{2\mu}$ optimally under the corresponding conditions at pH 4.5. This slightly higher pH was selected to avoid the random cleavage of rat IgM.

The author evaluated the immunoreactivity of $F(ab')_{2\mu}$ fragments by sandwich enzyme immunoassay (Fig. 6). In all cases, the loss of immunoreactivity were not found entirely (data not shown). Not surprisingly, therefore, purified $F(ab')_{2\mu}$ retained excellent immunoreactivity after preparation process. Furthermore, sandwich enzyme immunoassay for the quantitation of mIL-6 using $F(ab')_{2\mu}$ could drastically reduced non-specific binding as comparison of IgM (i. e., CY-04: IgM, 0.212; $F(ab')_{2\mu}$ 0.019). The detection limit for mIL-6 using the IgM mAb was 0.1 ng/ml and this was improved to 1 pg/ml using $F(ab')_{2\mu}$ fragments, which is similar to that reported for human IL-6 (6). The binding activity of $F(ab')_{2\mu}$ fragments to human C1q complement was lost completely as same as murine $F(ab')_{2\mu}$ (Fig. 5). The sensitivity and reproducibility of detection of mIL-6 were greatly improved (Fig. 6). The non-specific binding as well as the complement C1q binding of mAbs may be due, in part, to sugar moieties in Fc regions (11). In conclusion, the author believes that this procedure is a very useful addition to the repertoire of techniques available for the sandwich enzyme immunoassay using rat IgM mAbs.

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Chapter 2

Flow Cytometric Analysis of Sialyl Lewis ^{a} Antigen on Human Cancer Cells by Using $F(ab')_{2\mu}$ Fragments Prepared from a Mouse IgM Monoclonal Antibody

Introduction

Immuno-histochemical staining is useful for analysis of cancer cells because it allows us direct visualization of cancer-associated antigens on cell surfaces (1). Flow cytometry is widely applied for cancer diagnosis and classification of leukocytes, although it is restricted by monoclonal antibodies (mAb) which bind to the antigens (2).

Most of the hybridomas raised against cancer-associated antigens on cell surfaces so far reported are somehow IgM producers. IgM molecules have a molecular mass of about 1,000 kDa and considerably low solubility, and this limits their application. Some efforts have been paid in order to overcome the difficulty in application of IgM. Screening class switch variants producing IgG mAbs from IgM-producing cells (3) and preparing single-chain antibodies by gene engineering (4, 5) are the examples. The author has recently developed a convenient method for the preparation of $F(ab')_2$ fragments of 144-153 kDa from pepsin digests of mouse and rat IgM mAbs (6-8) and suggested that the term $F(ab')_{2\mu}$ should be used instead of $F(ab')_2$ to indicate that the fragments are prepared from IgM by cleaving the heavy μ chains. $F(ab')_{2\mu}$ fragments were demonstrated to be used suitably in enzyme-linked immunosorbent assay (ELISA), and the interaction of IgM mAb with non-specific proteins was greatly reduced, when it was converted to the fragments.

Sialyl Lewis ^{a} antigen (monosialyl, monofucosyllacto-*N*-tetraose; IV^{3}NeuAc, III^{4}Fuc-LcOse$_4$) is a well known tumor marker represented on cancer cells as glycolipids and glycoproteins (9-11) and is utilized for diagnosis of gastrointestinal and pancreatic cancer (12). The author has raised a mouse hybridoma line producing an IgM mAb of anti-sialyl Lewis ^{a}. In this chapter, the author describes that F(ab')$_{2\mu}$ fragments prepared from the IgM mAb is useful in flow cytometric analysis of sialyl Lewis ^{a} antigen represented on human cancer cells in comparison with the original IgM mAb.

Materials and Methods

Monoclonal antibody and cell lines

A mouse mAb SA23.2 (IgM class) specific to sialyl Lewis ^{a} antigen was used. A glycolipid fraction containing sialyl Lewis ^{a} antigen was purified from human meconium, and was used as immunogen to a BALB/c mouse after being absorbed to *Salmonella minnesota* (13). A hybridoma line secreting mAb SA23.2 was established by fusing spleen cells from the immunized mouse with mouse SP2/0-Ag14 myeloma cells (14). The hybridoma cells were injected into pristane-primed BALB/c mice, and were grown in ascites fluids (15, 16). The mAb was purified from the ascites by 60% ammonium sulfate saturation followed by gel filtration on TSKgel Toyopearl HW-55 (Tosoh, Japan) in 100 mM citrate buffer, pH 4.5 (17). The purity of the mAb was examined by SDS-PAGE under reducing and non-reducing conditions and by gel filtration HPLC using TSKgel G4000SW$_{XL}$. The specificity of mAb SA23.2 was examined by enzyme-linked immunosorbent assay (17). mAb SA23.2 binds to sialyl Lewis ^{a} antigen but not to asialo or

defucosylated forms of the antigen (see below, Immunoreactivity). The cell lines, Colo201 and SW1116 (human colon adenocarcinoma), Capan2 (human pancreas adenocarcinoma), MKN74 (human stomach adenocarcinoma), and QG56 and PC9 (human lung carcinoma) were supplied from the American Type Culture Collection (Rockville, MD), and routinely maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivate d FCS (Bioserum, Victoria, Australia), at 37°C in an atmosphere of 5% carbon dioxide.

Preparation of F(ab')$_{2\mu}$ fragments

F(ab')$_{2\mu}$ fragments of IgM mAb SA23.2 were prepared by the method previously reported (6). The IgM was digested with pepsin at a pepsin-to-IgM ratio of 1:200 (w/w) in 100 mM citrate buffer, pH 4.2, at 37°C for 2 h. F(ab')$_{2\mu}$ fragments were purified to the homogeneity by high-performance liquid chromatography (HPLC) using TSKgel Ether-5PW (Tosoh) (6).

Preparation of FITC-conjugates of mAb and F(ab')$_{2\mu}$

Conjugation of IgM and F(ab')$_{2\mu}$ with FITC (Dojindo, Kumamoto, Japan) was performed by the method previously reported (19, 20). The conjugates were separated from free FITC by gel filtration HPLC on a TSKgel G4000SW$_{XL}$ column (7.8 mm (inner diameter) x 30 cm) (Tosoh) with PBS (pH 7.4) at a flow-rate of 1 ml/min. The collected fraction was applied again to HPLC under the same conditions. The FITC-conjugates of mAb and F(ab')$_{2\mu}$ eluted in a single peak at 7.7 and 11.0 min, respectively. Fractions of 0.5 ml were collected and stored at 4°C in the dark with 0.05% (w/v) sodium azide until use. The concentration of mAb and F(ab')$_{2\mu}$ was determined by the absorptivity coefficient A_{280} (1 mg/ml) of 1.4, and that of FITC was by the molar absorptivity coefficient (495 nm) of 67,600. Number of

fluorescein moiety bound to the mAb and the $F(ab')_{2\mu}$ molecules were determined to be 4.9 and 1.2, respectively, by the following equation (21):

FITC / protein molar ratio = 2.87 x A_{495} / (A_{280}-0.35 x A_{495}). by using the absorptivities of the conjugates at 280 and 495 nm.

Preparation of alkaline phosphatase-conjugates of mAb and $F(ab')_{2\mu}$

Bovine intestine alkaline phosphatase (type VII-S) was purchased from Sigma (St. Louis, MO). A 2-ml enzyme solution (2 mg/ml) in PBS was incubated with 20 μl of *N*-succinimidyl-3-(2'-pyridyldithio)propionate (SPDP) (5 mg/ml) at 4°C for 16 h, followed by dialysis at 4°C. A 2-ml solution of mAb or the fragments (2 mg/ml) in PBS was treated 30 μl *S*-acetylmercapto-succinic anhydride (SAMSA) (10 mg/ml) at 37°C for 1 h, followed by dialysis at 4°C. The alkaline phosphatase treated with SPDP and the mAb or $F(ab')_{2\mu}$ treated with SAMSA were mixed with 50 μl of 1 M NH_2OH at 4°C for 1 day. The reaction mixture was applied to gel filtration HPLC on a TSKgel G3000SW_{XL} (7.8 mm (inner diameter) x 30 cm) at a flow-rate of 1.0 ml/min, and the conjugates were separated from the free enzymes. The elution was detected by absorption at 280 nm.

The numbers of the enzyme molecules bound to the mAb and $F(ab')_{2\mu}$ fragment were determined to be 3.5 and 1.1 (mole/mole), respectively, by gel filtration HPLC. The molecular mass of the enzyme-$F(ab')_{2\mu}$ conjugate was estimated to be 260 kDa, suggesting that 1.1 moles of the enzyme (dimer of 50 kDa subunits) were conjugated with a mole of the fragment (145 kDa). When the same amount of $F(ab')_{2\mu}$ in the free state or in the enzyme-$F(ab')_{2\mu}$ conjugate was applied to gel filtration HPLC, the peak area of the conjugate was 1.8 times larger than that of the free $F(ab')_{2\mu}$. This means that the molecular mass of the conjugate is 1.8 times larger than that of the fragment, by assuming the same

absorptivity coefficient, A_{280} (1 mg/ml) for both $F(ab')_{2\mu}$ and enzyme-$F(ab')_{2\mu}$ conjugate. This leads to an estimation that 1.2 moles of the enzyme (dimer of 50 kDa subunits) are conjugated to a mole of $F(ab')_{2\mu}$. The molar ratio of the enzyme to $F(ab')_{2\mu}$ is in good agreement with the value 1.1 estimated from the elution volume in HPLC.

The enzyme-mAb conjugate as well as the mAb itself eluted at the void volume in the gel filtration HPLC, being separated from the free enzyme. As the molecular mass of the conjugate could not be estimated from the elution volume, the peak areas in the gel filtration HPLC of the same amount of mAb in the free state or in the enzyme-mAb conjugate were compared. The peak area of the conjugate was 1.35 times larger than that of the free mAb. By assuming the absorptivity coefficient, A_{280} (1 mg/ml) of the mAb and the conjugate to be the same, the molecular mass of the conjugate was estimated to be 1,350 kDa, suggesting that 3.5 moles of the enzyme were conjugated with one mole mAb.

The enzyme activity of the conjugates was examined in the hydrolysis of pNPP in 50 mM carbonate buffer (pH 9.5) containing 10 mM $MgCl_2$. Concentrations of the conjugates and the enzyme were determined by using an absorptivity coefficient, A_{280} (1 mg/ml) of 1.4, and molecular masses of the enzyme (100 kDa), enzyme-mAb conjugate (1,350 kDa) and enzyme-$F(ab')_{2\mu}$ conjugate (260 kDa). The catalytic constants *k*cat and Michaelis constant *K*m of the enzyme was determined to be 48 s^{-1} and 1.8 mM, respectively; those of the enzyme-mAb conjugate were 123 s^{-1} and 1.8 mM, and of enzyme-$F(ab')_{2\mu}$ conjugate 22 s^{-1} and 2.0 mM, respectively. As the enzyme-mAb and enzyme-$F(ab')_{2\mu}$ conjugates contain 3.5 and 1.1 enzyme moles in a mole of the conjugates, the *k*cat values calculates on the basis of enzyme concentration might be 35 and 20 s^{-1}, respectively. Namely, the catalytic activities of the enzyme bound to the mAb and $F(ab')_{2\mu}$ are 73% and 42% of that of the free enzyme, respectively, although the *K*m values are not

changed by the conjugation.

Immunoreactivity

Immunoreactivity of mAb SA23.2 and its $F(ab')_{2\mu}$ fragments against CA19-9, a serum mucin containing sialyl Lewis ^{a} antigen, was measured by means of sandwich enzyme immunoassay. CA19-9 was obtained from Centocor (Malvern, PA) and determined by using their radioimmunoassay kits.

A microtiter plate (96 wells; Nunc-Intermed, MaxiSorp; Roskilde, Denmark) was coated with mAb SA23.2 (100 μl of 2 μg/ml in 50 mM sodium carbonate buffer, pH 9.5, in each well) and incubated at 37°C for 1 h. Plates were blocked by incubation with 0.2% bovine serum albumin (BSA) in PBS (pH 7.4) overnight at 4°C. After washing the plate with PBS, the plate was incubated with CA19-9 at various concentrations (0, 25, 50, 100, 200, and 400 U/ml) in 100 mM PBS containing 0.2% BSA at 37°C for 1 h. The plate was washed again with PBS and incubated with 100 μl of 2 μg/ml alkaline phosphatase conjugates with mAb SA23.2 or $F(ab')_{2\mu}$ fragments at 37°C for 1 h. The plate was washed again with PBS, followed by adding 100 μl of the substrate solution (1 mg/ml pNPP in 50 mM carbonate buffer, pH 9.5, containing 10 mM $MgCl_2$). Absorbance at 405 nm was measured after the reaction for 20 min at 37°C.

The specificity of mAb SA23.2 was examined by inhibition assay with three carbohydrate antigens (Table 1), monosialyl, monofucosyllacto-*N*-tetraose (IV^3NeuAc, III^4Fuc-$LcOse_4$), lacto-*N*-fucopentaose II (III^4Fuc-$LcOse_4$), and sialyllacto-*N*-tetraose a (IV^3NeuAc-$LcOse_4$), purchased from BioCarb Chemicals (Lund, Sweden) (18). A hundred μl of the antigen at various concentrations (0, 0.6, 5, 25, 100, and 500 μg/ml) was added to the mAb SA23.2-coated well prepared as above, and incubated with 100 μl of 2 μg/ml

Table 1 Structure of carbohydrate antigen

Monosialyl, monofucosyllacto-*N*-tetraose (sialyl Lewis^a)

Neu5Ac α 2-3Gal β 1-3GlcNAc β 1-
4
I
Fuc α 1

Sialyllacto-*N*-tetraose a

Neu5Ac α 2-3Gal β 1-3GlcNAc β 1-

Lacto-*N*-fucopentaose II

Gal β 1-3GlcNAc β 1-
4
I
Fuc α 1

enzyme-mAb conjugate and 240 U/ml of CA19-9 at 37°C for 1 h. The specificity of mAb SA23.2 was measured by means of the sandwich enzyme immunoassay described above. The inhibitory activity of the carbohydrate antigen was estimated by comparing the absorbance at 405 nm observed in the presence of the carbohydrate antigen (inhibitor) with that observed in the absence.

Immunofluorescent staining

For staining of Colo201, SW1116, Capan2, MKN74, PC9, and QG56 cells, 20 μl of FITC conjugates of mAb SA23.2 or its $F(ab')_{2\mu}$ fragments was mixed with 10^6 cells suspended in 100 μl of PBS containing 10% FCS, and incubated at 37°C for 10 min. Free conjugates were removed by washing the cells twice with PBS by centrifugation at 4000 x g for 5 min. The cells were resuspended again in PBS, and applied to flow cytometry at 37°C.

Flow cytometry

Flow cytometry was performed using a FACStar^{plus} flow cytometry (Becton Dickinson, San Jose, CA) equipped with an argon laser and a FACStar research software. Cells were excited at 488 nm and FITC emission was detected with a 530±30 nm bandpass filter (Fig. 1 and 2). Fluorescence data were displayed on four-decade log scales. Ten thousands or more data were collected for each analysis.

Results and Discussion

Immunoreactivity

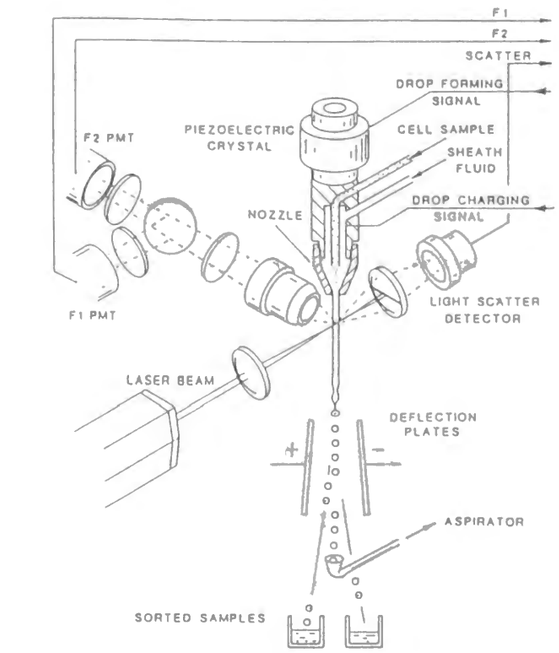


Fig. 1. Generalized cell sorter diagram (Parks et al., 1986). The functions of the components in the mechanical and optical systems.

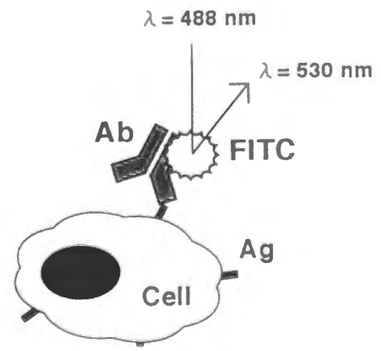


Fig. 2. Schematic representation of flow cytometry. Cells were excited at 488 nm and FITC emission was detected with a 530±30 nm bandpass filter. Fluorescence data were displayed on four-decad e log scales. Ten thousands or more data were collected for each analysis.

Immunoreactivity of mAb SA23.2 and the $F(ab')_{2\mu}$ fragments to CA19-9 antigen is shown in Fig. 3. A standard curve was obtained in the CA19-9 concentration ranging from 0 to 400 U/ml. The immunoreactivity of mAb SA23.2 is 1.45 times larger than that of the $F(ab')_{2\mu}$ fragments. As all measurements of enzyme activity were carried out under the conditions that substrate concentration was in large excess over the Michaelis constant *K*m (1.8 mM), the reaction velocity must be the maximum velocity, *V*max. Assuming that the same amount of enzyme was bound to the CA19-9 antigens captured on the plate when the enzyme conjugates of mAb and $F(ab')_{2\mu}$ fragments were used as the 2nd antibody, the enzyme activity of the enzyme-mAb conjugate would be 1.75 times larger than that of enzyme-$F(ab')_{2\mu}$ conjugate. Therefore, enzyme molecules bound onto the plates with the enzyme-mAb conjugates could be 83% of that with the enzyme-$F(ab')_{2\mu}$ conjugate and that the number of the $F(ab')_{2\mu}$ fragments bound onto the plate is 3.8 times larger than that of the mAb.

Figure 4 demonstrates that monosialyl, monofucosyllacto-*N*-tetraose inhibits the binding of mAb SA23.2 with CA19-9, however, lacto-*N*-fucopentaose II and sialyllacto-*N*-tetraose a show no significant inhibition. The similar inhibitory effect was observed on the binding between $F(ab')_{2\mu}$ and CA19-9 (data not shown). This indicates that both sialyl and fucosyl groups of sialyl Lewis ^{a} antigen are essential in the recognition of CA19-9 by mAb SA23.2 and the $F(ab')_{2\mu}$ fragments.

Flow cytometry

Flow cytometry of MKN74, QG56 and PC9 cells, being sialyl Lewis ^{a} negative, was examined by staining them with FITC conjugates of mAb SA23.2 and its $F(ab')_{2\mu}$ fragments. No staining of PC9 (Fig. 5A) and MKN74 cells (data not shown) was observed

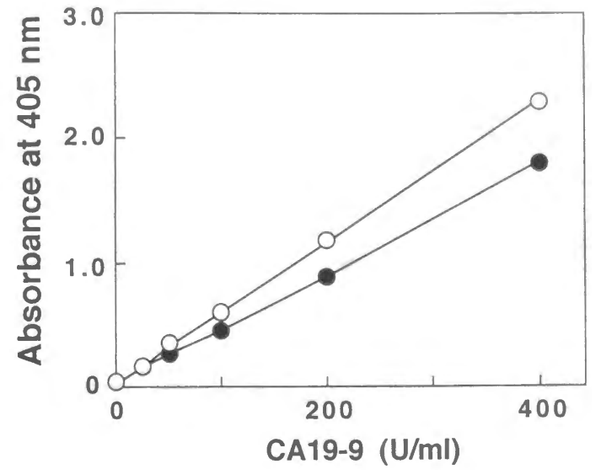


Fig. 3. The immunoreactivity of IgM mAb SA23.2 and its $F(ab')_{2\mu}$ fragments with CA19-9. Each well of a 96-well microtiter plate was coated with mAb SA23.2 by adding 100 μl mAb SA23.2 solutions (2 μg/ml). A hundred μl of CA19-9 solution at the concentration indicated on the abscissa was added to the well, followed by the addition of 100 μl of 2 μg/ml alkaline phosphatase conjugates of IgM mAb SA23.2 (○) and its $F(ab')_{2\mu}$ fragments (●). Absorbance at 405 nm generated by the reaction with p -nitrophenyl phosphate for 20 min at 37°C was observed. Mean values of the tripricate measurements were plotted.

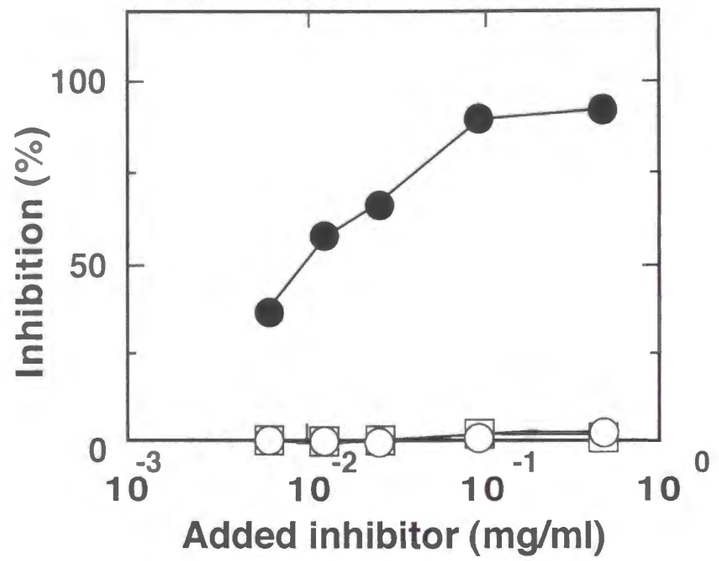


Fig. 4. Inhibition of the immunoreactivity of mAb SA23.2 against CA19-9 by carbohydrate antigens. Each well of 96-well microtiter plate was coated with mAb SA23.2 by adding 100 μl mAb SA23.2 solution (2 μg/ml). A hundred μl of a carbohydrate antigen solution at the concentration indicated on the abscissa was added to the well with alkaline phosphatase conjugate of mAb SA23.2 (2 μg/ml) and CA19-9 (249 U/ml), and incubated at 37°C for 1 h. Absorbance at 405 nm generated by the reaction with *p* -nitrophenyl phosphate for 20 min at 37°C was observed. Carbohydrate antigen: monosialyl, monofucosyllacto-N -tetraose (IV^3NeuAc, $III^4Fuc\text{-}LcOse_4$), ●; sialyllacto-N -tetraose a ($IV^3NeuAc\text{-}LcOse_4$), ○; and lacto-N -fucopentaose II ($III^4Fuc\text{-}LcOse_4$), □. Inhibition (%) was estimated as [1- (A_{405} in presence of the inhibitor) / (A_{405} in the absence of the inhibitor)] x 100.

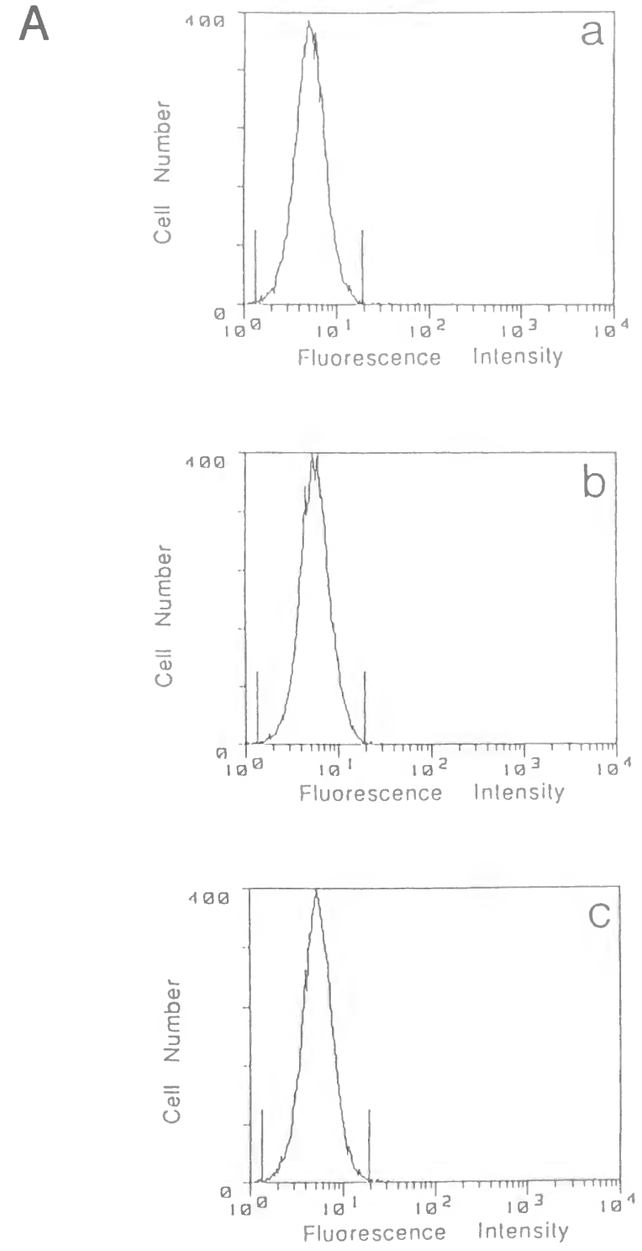
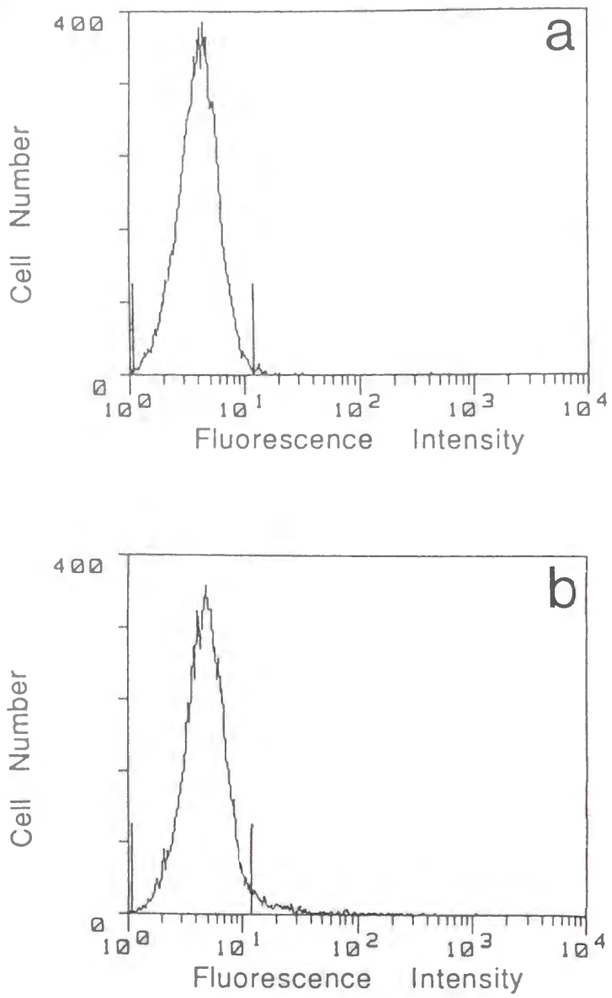


Fig. 5. Flow cytometric analysis of cancer cells with FITC conjugates of mAb SA23.2 and its $F(ab')_{2\mu}$ fragments. One million cells were incubated with 5 μg/ml of FITC-mAb or FITC-$F(ab')_{2\mu}$ conjugates in 100 μl at 37°C for 10 min, and washed by PBS twice before flow cytometry. Cells were excited at 488 nm and FITC emission was detected with a 530±30 nm bandpass filter. A, PC9 human lung carcinoma cells; B, QG56 human lung carcinoma cells; and C, Colo201 human colon adenocarcinoma cells. Cells were incubated with a, PBS; b, FITC-$F(ab')_{2\mu}$; and c, FITC-mAb conjugates.

B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Cell Number
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Fluorescence Intensity

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Fluorescence Intensity

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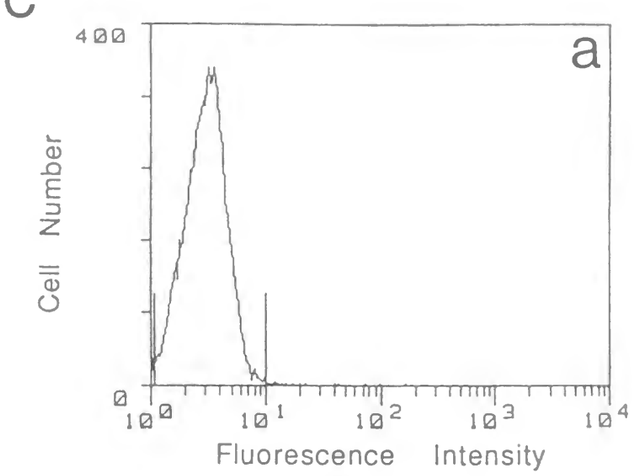
Fluorescence Intensity

C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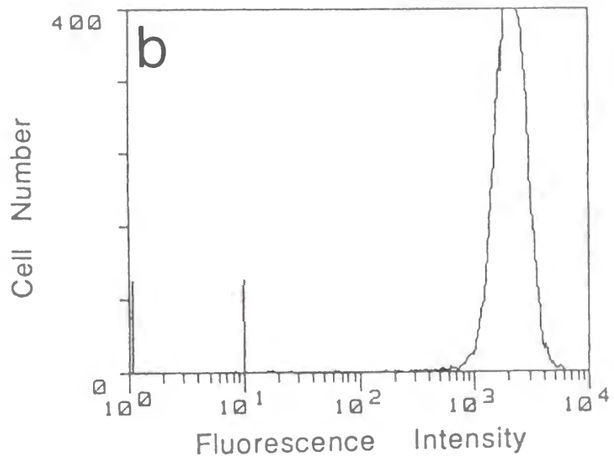
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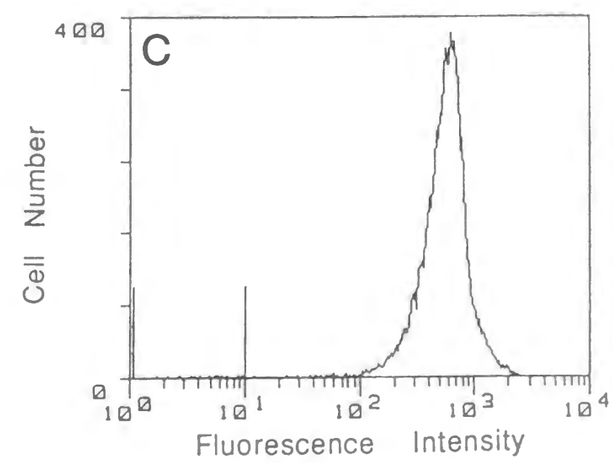
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10⁰ 10¹ 10² 10³ 10⁴
Fluorescence Intensity

with both conjugates, and for QG56 cells (Fig. 5B), 3 and 25% of the total population was stained with FITC-F(ab')$_{2\mu}$ and FITC-mAb conjugates, respectively. It was also shown that 20-25% of the QG56 cell population was stained even with the FITC conjugates of non-specific IgM mAbs (data not shown). This suggests that mAb SA23.2 binds QG56 cells non-specifically, and the non-specific binding is reduced drastically from 25 to 3% by using F(ab')$_{2\mu}$ fragments in place of IgM mAb. Figure 5C shows the staining of Colo201 cells, which are sialyl Lewis ^{a} positive, by FITC conjugates of mAb SA23.2 and the F(ab')$_{2\mu}$ fragments. All Colo201 cells were stained with both conjugates, and the fluorescence intensity induced with FITC-F(ab')$_{2\mu}$ was 5 times higher than that with FITC-mAb. Numbers of FITC molecules conjugated on mAb and F(ab')$_{2\mu}$ fragments are 4.9 and 1.2, respectively. Relative fluorescence intensities of FITC conjugates of mAb and F(ab')$_{2\mu}$ were almost proportional to the numbers of FITC molecules carried on the respective proteins, and thus the quantum yield of FITC on both molecules is considered to be reasonably the same. If the same numbers of mAb and F(ab')$_{2\mu}$ molecules were bound to the cells, fluorescence intensity of FITC conjugated with mAb might be 4.1 times higher than that with F(ab')$_{2\mu}$, although it was actually 1/5. Accordingly, the number of F(ab')$_{2\mu}$ molecules bound on the cells is estimated to be 21 times more than that of mAb. These lines of evidence suggest that the FITC-mAb conjugate cannot bind densely to the CA19-9 antigens expressed on the surface of Colo201, probably because of its large size and steric hindrance. On the other hand, the FITC-F(ab')$_{2\mu}$ conjugate, which is 1/5 in size of the FITC-mAb, can bind the cells more densely than the FITC-mAb. Distribution of the cells stained with FITC-F(ab')$_{2\mu}$ was narrower than that with FITC-mAb, suggesting that the binding of F(ab')$_{2\mu}$ fragments to Colo201 cells is stronger and more specific than that of mAb SA23.2 (Fig. 5C). Flow cytometric analysis of SW1116 and Capan2 showed the

similar results to that of Colo201. These cells were stained by both FITC conjugates of mAb and F(ab')$_{2\mu}$. The fluorescence intensities of SW1116 and Capan2 cells induced with the FITC-F(ab')$_{2\mu}$ conjugates were 5 and 4 times higher than that with the FITC-mAb conjugates, respectively.

Usefulness of F(ab')$_{2\mu}$ fragments in the enzyme immunoassays

F(ab')$_{2\mu}$ fragments have been shown to be superior to their original IgM mAb as a staining reagent of sialyl Lewis ^{a} antigen on cell surfaces by means of flow cytometry. This might be explained by several reasons. F(ab')$_{2\mu}$ fragments may bind the antigens expressed on cellular membranes more easily than IgM because of their smaller molecular size; 145 kDa for the fragments and 1,000 kDa for IgM. The sugar content of F(ab')$_{2\mu}$ fragments is much reduced as compared with that of IgM, because of the loss of the Fc region being abundant in sugar residues, which may lead to the less non-specific binding (6, 8). The binding of IgG and IgM mAbs to non-specific proteins in enzyme immunoassay is greatly reduced by using the F(ab')$_2$ fragments instead of the whole mAb molecules (6, 8, 15). In this chapter, it has been also demonstrated that the smaller molecular size and less sugar content of F(ab')$_{2\mu}$ fragments seem to make them more useful as a staining reagent in flow cytometry. Recently, single-chain antibody containing only the Fv region of mAb has been expressed in *E. coli*, the molecular size of which is around 20 kDa and the sugar content is zero (4, 5). However, preparing single-chain antibody requires genetic engineering techniques, and it is time-consuming compared to the preparation of F(ab')$_2$ fragments. F(ab')$_{2\mu}$ fragments provides us with the higher sensitivity and specificity in flow cytometric analysis than IgM mAb, and they can be useful to determine the antigens presented on cell surfaces.

Conclusion

Monoclonal antibodies are of great interest in diagnosis and therapy. IgG mAbs are mainly used in practice, and IgMs have been avoided as possible because of their difficulties in handling. mAbs raised against cancer-associated sugar antigens could be useful for cancer diagnosis and therapy, however, almost all mAbs so far reported are of IgM-class. The author has reported a convenient method for preparing active fragments of IgM, and showed that the fragments could be applied to ELISA better than IgM (6-8).

$F(ab')_{2\mu}$ fragments prepared from mouse IgM mAb SA23.2 specific to sialyl Lewis ^{a} antigen were applied to flow cytometry to analyze the antigen on human cancer cells. The binding of the fragments to the antigen-positive cells was stronger than that of the original IgM, and the non-specific binding of the IgM antibody to the antigen-negative cells was much decreased by using the $F(ab')_{2\mu}$ fragments. These results indicate that the $F(ab')_{2\mu}$ fragments are more suitable than the original IgM mAb in flow cytometry as well as ELISA.

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Chapter 3

A Sensitive Enzyme Immunoassay of Human Thyroid-stimulating Hormone (TSH) by Using Bispecific F(ab')$_2$ Fragments Recognizing Polymerized Alkaline Phosphatase and TSH

Introduction

Bispecific monoclonal antibody (bsmAb) is the immunoglobulin molecules with ability to bind two different antigen simultaneously. They are convenient tools for immunohistochemistry (1), immunotherapy (2-4), and immunodiagnosis (5, 6). bsmAb can be obtained by three methods: cell fusion of two different hybridomas (1, 7-10), biochemical linkage of two different Fab' fragments of monoclonal antibodies (11, 12), and genetic manipulations (13).

Although hybrid hybridomas constantly secrete bsmAb, they are highly polyploid and exhibit a higher propensity to lose chromosomes (9). In addition, they secrete a mixture of both bsmAb and parental antibodies are produced. bsmAbs were produced by the recombination of F(ab)SH in presence of 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) (11) or homobifunctional crosslinking reagent (*o*-phenylene dimaleimide) (12). These studies showed that the specificity and affinities of the bsmAb are almost same as those of the parental monoclonal antibodies.

The yield of chemically prepared bsmAb have been reported between 50 to 70% (11),

and it was deeply dependent upon preparation of F(ab')$_2$ of IgG1 by pepsin digestion. The high yields of F(ab')$_2$ by pepsin digestion reported by Morimoto and Inouye (14) were 42-58% without any loss of immunoreactivity. This method may be well applied to preparation of bispecific F(ab')$_2$.

BsmAbs have been purified from parental mAb or other inactive antibody using ion exchange chromatography (7), two-step affinity chromatography (15), or hydroxylapatite chromatography (16). It is significantly difficult to separate the bsmAb from other antibodies, and contaminant antibody which strongly interfere with the antigen binding of bsmAb.

Previous studies have demonstrated that bsmAb could be used in one-step immunoassay by the binding ability to enzyme antigen and other antigen (7, 9, 17-19). Inactivation of mAb and enzyme due to covalent linkage could be avoided by the use of bispecific mAb. Actually, immunoassay using bsmAb showed almost the same sensitivities comparing with that using enzyme-mAb conjugates. bsmAbs do not suffer any disadvantages, and they have allowed us to develop a sensitive sandwich ELISA method.

The sensitivity of ELISA is dependent on the enzyme activity of enzyme-mAb conjugates. If polymerized enzyme is used to label the mAb without the loss of activity, the sensitivity might increase according to the degree of polymerization.

In this chapter, bsmAb were clearly purified from parental Fab' fragments by using hydrophobic interaction HPLC procedure, and a new one-step sandwich ELISA method was established by using bsmAb and alkaline phosphatase polymers. bsmAb was prepared by the method of Brennan et al. (11) from anti-ALP and anti-human thyroid stimulating hormone (TSH) monoclonal antibody. Polymerized ALP was prepared by glutaraldehyde coupling.

Materials and Methods

Monoclonal Antibodies

Four mouse mAbs APA03, APA05, TSA07, and TSA08 of the IgG1 class were used. The specific antigen of APA03 an APA05 is bovine calf intestine ALP (EC 3.1.3.1.) and that of mAb TSA07 and TSA08 is human TSH. Hybridomas secreting the mAbs were established in our laboratory, by fusing spleen cells from an antigen-immunized BALB/c mouse with SP2/0-Ag14 myeloma cells according to Köhler and Milstein (20). The hybridoma cells were injected into pristane-primed BALB/c mice, and were grown in ascites fluids. The mAbs were purified from the ascites by 60% ammonium sulfate saturation followed by gel filtration on TSKgel Toyopearl HW-55 (Tosoh, Tokyo, Japan) in 100 mM sodium citrate buffer, pH 4.5 (21). Concentration of mAb was determined spectrophotometrically using absorbance at 280 nm, A_{280} (1 mg/ml) of 1.4.

Bovine intestinal ALP (Type VII-S) was purchased from Sigma (St. Louis, MO), and TSH of human pituitary glands (standard grade) was from Scripps Laboratories (San Diego, CA).

HPLC

The HPLC apparatus consisted of a solvent-delivery system CCPM, a UV monitoring system UV-8010, a fraction collector FC-8000, and a computer-control system SC-8010 was purchased from Tosoh (Tokyo). The HPLC was performed at a flow-rate of 1 ml/min at room temperature; the elution was monitored by A_{280}; and fractions (1 ml) were collected. Hydrophobic interaction HPLC was performed on TSKgel Ether-5PW or Phenyl-5PW

columns (7.5 mm (inner diameter) x 75 mm) (Tosoh), and gel-filtration HPLC was using a TSKgel G3000SW$_{XL}$ column (7.8 mm (inner diameter) x 30 cm).

SDS-PAGE

SDS-PAGE was performed in a 8-16% gradient slab gel (TEFCO, Tokyo) under non-reducing condition, according to the method of Laemmli (22). The molecular mass marker kit consisting rabbit muscle myosin (200 kDa), *Escherichia coli* β-galactosidase (116 kDa), bovine serum albumin (BSA) (66 kDa), rabbit muscle aldolase (42 kDa), bovine erythrocyte carbonic anhydrase (30 kDa), and horse muscle myoglobin (17 kDa) is a product of Daiichi Chemicals (Tokyo). Proteins were stained with Coomassie brilliant blue R-250.

Preparation of $F(ab')_2$ fragments by pepsin digests

Pepsin digestion of mAbs was carried out according to the method of Morimoto and Inouye (14). The purified mAb was digested by porcine pepsin (EC 3.4.23.1) (Sigma, St. Louis, MO). The starting concentration of mAb was 1-5 mg/ml in 100 mM sodium citrate buffer (pH 3.5). Pepsin was added to the mAb solution at a weight ratio of 1:100 (pepsin:IgG1). Digestion proceeded at 37°C for 2 h, and was stopped by adding 3 M tris(hydroxymethyl)aminomethane (Tris) to give a pH around 7. The pepsin digests were salted out with 60% saturated ammonium sulfate, and the precipitates were dissolved in PBS (pH 7.4) containing 1 M ammonium sulfate. The solution was applied to the hydrophobic interaction HPLC on a TSKgel Phenyl-5PW column equilibrated with PBS containing 1 M ammonium sulfate (pH 7.4), and eluted with a linear gradient of ammonium sulfate from 1 to 0 M in PBS (pH 7.4), for 30 min. Purity of $F(ab')_2$ fragments were checked

by gel filtration HPLC on a TSKgel G3000SW$_{XL}$ column.

Bispecific F(ab')$_2$ fragments

Bispecific F(ab')$_2$ fragments were prepared by the modified method of Brennan et al. (11). F(ab')$_2$ fragments of anti-ALP mAbs were reduced to Fab'-SH with 10 mM dithiothreitol in PBS containing 5 mM EDTA, and applied to gel-filtration HPLC on a TSKgel G3000SW$_{XL}$ column equilibrated with the same buffer. Fab'-SH were derivated to thionitrobenzoate (TNB) in 10 mM acetic acid (pH 5.5) containing 5 mM EDTA, and excess DTNB was remove with a TSKgel G3000SW$_{XL}$ column. Fab'-TNB of anti-ALP mAb were mixed with Fab'-SH of anti-TSH mAb (TSA07) at a molar ratio of 2:1, and incubated for 3 days at 4°C under nitrogen gas.

Purification of bsmAb

Hydrophobic interaction HPLC was performed on a TSKgel Ether-5PW column (7.5 mm (inner diameter) x 75 mm) (Tosoh). bsmAbs were dialyzed against PBS containing 1.5 M ammonium sulfate (pH 7.4) for 3 h at 4°C. The solution was applied to the column equilibrated with the same buffer, and eluted with a linear gradient of ammonium sulfate from 1.5 to 0 M in PBS, for 30 min at a flow-rate of 1 ml/min at room temperature, and was collected 1 ml fractions of effluent.

Assays of antibody activities

Detection of anti-ALP antibody activity

A microtiter plate (96 wells; Nunc-Intermed, MaxiSorp; Roskilde, Denmark) was coated with 100 µl goat anti-mouse IgG polyclonal antibody (Nordic Immunological Laboratories,

Tilburg, The Netherlands; 5 µg/ml in PBS) to each well, followed by incubation for 2 h at 25°C. Plate was blocked by incubation with 0.1% BSA in PBS overnight at 4°C. After washing the plate once more with PBS, the fractions (50 µl) of bispecific F(ab')$_2$ eluted from the Ether-5PW column and 50 µl of ALP (5 µg/ml in PBS) were added to the plate, and incubated for 2 h at 25°C. The plate was washed with PBS, followed by the addition of 100 µl of 1.0 mg/ml substrate (pNPP in 50 mM carbonate buffer, pH 9.5 containing 10 mM $MgCl_2$) for 30 min at 25°C. The reaction was terminated by adding 200 µl of 1 N NaOH, and absorbance at 405 nm was measured by a microtiter plate reader MPR-A4 (Tosoh).

Detection of anti-TSH antibody activities

A microtiter plate was coated with 100 µl TSH (5 µg/ml in PBS) to each well, followed by incubation for 2 h at 25°C, and blocked by incubation with 0.1% BSA in PBS overnight at 4°C. The plate was washed with PBS, and the fractions (50 µl) of bispecific F(ab')$_2$ eluted from the Ether-5PW column were added and incubated for 2 h at 25°C. After washing the plate with PBS again, excess goat anti-mouse IgG Ab conjugated with horseradish peroxidase (1/5,000; 50 µl; Jackson Immunoresearch Laboratories, West Grove, PA) was added and incubated for 2 h at 25°C. The plate was washed with PBS, then the enzyme reaction was started by adding 100 µl substrate (0.2 mg/ml ABTS and 0.03% H_2O_2 in 100 mM citrate buffer, pH 4.1) to each well, and terminated by 100 µl oxalic acid (0.1 M) after the reaction for 30 min at 25°C. Absorbance at 415 nm was measured.

Preparation of polymerized ALP

Bovine intestine ALP (Type VII-S) was purchased from Sigma (St. Louis, MO), and the concentration was determined spectrophotometrically by A_{280} (1 mg/ml) of 0.78. ALP (5 mg/ml) was dialyzed thoroughly against 100 mM PBS, and mixed with 0.02% glutaraldehyde in 50 mM Tris-HCl buffer (pH 7.4) for 16 h at 4°C (23). The reaction was terminated with the addition of 0.1 M L-lysine. Following dialysis against the same buffer for 6 h at 4°C, the reaction mixture was applied to gel-filtration HPLC on a TSKgel G3000SW$_{XL}$ column equilibrated with the Tris-HCl buffer.

One-step sandwich ELISA for TSH

The immunoreactivity of bsmAb was measured by means of sandwich enzyme immunoassay. Microtiter plates were coated with F(ab')$_2$ of TSA08 mAb (100 μl of 3 μg/ml in 50 mM sodium carbonate buffer, pH 9.5, in each well) and incubated for 1 h at 37°C. The plates was blocked by incubation with 0.1% BSA in PBS overnight at 4°C. After the plate was washed with PBS, the plate was incubated with TSH at various concentrations (0, 25, 50, and 100 μU/ml) in 100 μl of 0.1% BSA in PBS, 50 μl of bispecific F(ab')$_2$ (APA05-TSA07) (A_{280}=0.001; 0.75 μg/ml), and 50 μl of polymerized ALP fractions (15 μg/ml) for 1 h at 37°C. A covalently linked ALP-TSA07 mAb conjugate prepared by the method of Jeanson et al. (24) was used as a reference, and 150 μl of the solution in PBS (A_{280}=0.03) was added to each well. The plate was washed again with PBS, followed by the addition of 100 μl of pNPP and measured at 405 nm as described for assay of antibody activity.

Results

Preparation of F(ab')$_2$ fragments from mAbs

The recovery yield of F(ab')$_2$ fragments, APA03, APA05, TSA07, and TSA08 were .048, 0.58, 0.56, and 0.52, respectively. These F(ab')$_2$ were eluted as a single peak, and the purity was estimated to be more than 98% by gel filtration HPLC (data not shown). These values are correspondent well with those reported previously (14).

Purification and detection of bispecific F(ab')$_2$ fragments

The Fab'-TNB of APA03 and APA05 were recombined to the free sulfhydryl form Fab'-SH of TSA07 mAb according to the modified method of Brennan et al. (11). Using hydrophobic interaction HPLC with Ether-5PW column (Fig. 1), the author has separated bsmAbs from unreacted Fab'-TNB(APA03 and APA05) and Fab'-SH(TSA07). Fig. 1 showed three major peaks, and their immunoreactivities were examined. When Fab' (APA03)-TNB and Fab'(TSA07)-SH were applied to the HPLC individually, the former eluted at 18 min, and the latter at 26 min (data not shown). Fractions of peak 1 showed immunoreactivity against ALP but not TSH, and those of peak 3 vice versa, indicating that proteins eluted at peaks 1 and 3 are Fab'(APA03)-TNB and Fab'(TSA07)-SH, respectively. On the other hand, proteins eluted in peak 2 showed immunoreactivity against both antigens (Fig. 1B), suggesting that the proteins in peak 2 is the bispecific F(ab')$_2$ fragments formed by chemical linkage between Fab'(APA03)-TNB and Fab'(TSA07)-SH (hereafter designated as F(ab')$_2$(APA03-TSA07). Elution pattern of the reaction mixture of Fab' (APA05)-TNB and Fab'(TSA07)-SH showed also three major peaks 1, 2 and 3 at 15, 23, and 26 min, respectively (Fig. 1C). Fab'(APA05)-TNB and Fab'(TSA07)-SH were indicated to elute in peaks 1 and 3, and the bispecific F(ab')$_2$ fragments, F(ab')$_2$(APA05-TSA07), in peak

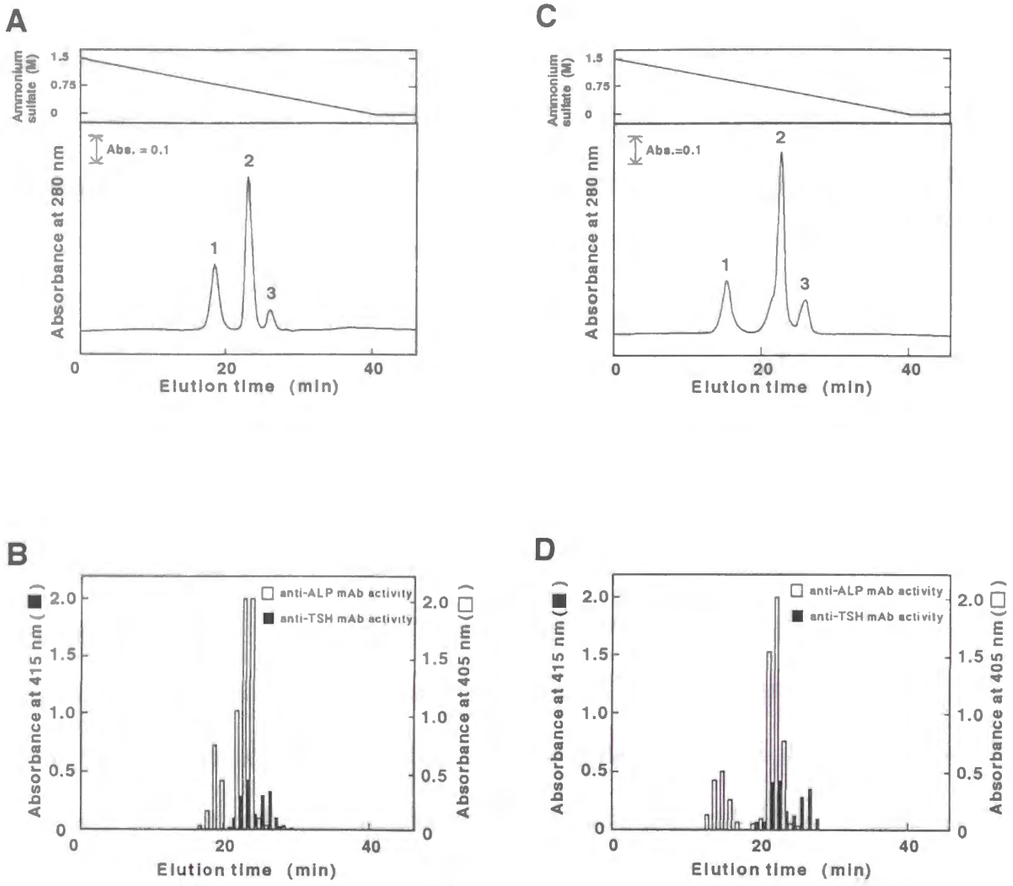

Fig. 1. Purification of bispecific $F(ab')_2$ fragments by hydrophobic interaction HPLC with TSKgel Ether-5PW and their immunoreactivities against ALP and TSH. The mixture of Fab' (APA03)-TNB and Fab' (TSA07)-SH (A and B) and that of Fab' (APA05)-TNB and Fab' (TSA07)-SH (C and D) were applied to the HPLC. Elution was monitored by measuring absorbance at 280 nm (A and C). Immunoreactivities of the fractions (1 ml) against ALP and TSH were examined by measuring absorbance at 415 nm (closed bars) and at 405 nm (open bars), respectively (B and D).

2 (Fig. 1D). The yields of the bispecific $F(ab')_2$ fragments prepared from the parental $F(ab')_2$ fragments were 65-70% of the theoretical values in both cases.

SDS-PAGE under non-reducing condition (Fig. 2), the bispecific $F(ab')_2$ fragments (lane 5, and 6) purified by hydrophobic interaction HPLC and $F(ab')_2$ of TSA07 (lane 7) showed a similar migration pattern. IgG1 mAbs (150-160 kDa) are converted to $F(ab')_2$ (110 kDa) in the pepsin digestion, where the heavy chain (50 kDa) is truncated to 30-kDa chain and the light chain is not degraded (14). Therefore, bispecific $F(ab')_2$ fragments of 110 kDa is considered to be composed of four chains; two pairs of a truncated heavy chain and an intact light chain derived from the anti-ALP and anti-TSH mAbs.

The yields of bispecific $F(ab')_2$ fragments from each parental $F(ab')_2$ were estimated 55-70% of the theoretical values in both cases.

Preparation of alkaline phosphatase polymers

A chromatogram on TSKgel G3000SW$_{XL}$ column of polymerized ALP showed three peaks at 7.0 min (peak 1), 7.7 min (peak 2), and 9.0 min (peak 3) (Fig. 3). Elution of peak 3 is in good agreement with that of the native ALP, which is a homodimer of 50-kDa subunits. Molecular mass values of the proteins eluted in the respective peaks were estimated to be 300, 200, and 100 kDa from the elution of standard proteins. The activity of anti-ALP antibody are recognized peak 1 and peak 2. The activity of alkaline phosphatase were compared with one-step sandwich ELISA of TSH.

Sandwich ELISA using bsmAb and alkaline phosphatase polymers

Affinity constants of APA05 and APA03 mAbs against ALP were determined to be 2.0 and 12 nM, respectively (data not shown). Because of the higher affinity of APA05 mAb

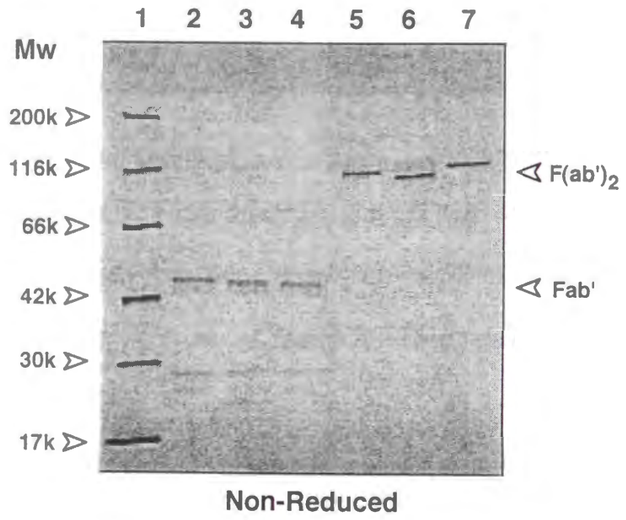

Fig. 2. SDS-PAGE of bispecific $F(ab')_2$ fragments under non-reducing conditions. Lane 1: molecular mass marker, Lane 2, 3 and 4: Fab'(APA03)-TNB, Fab'(APA05)-TNB, and Fab'(TSA07)-SH fragments, respectively; Lane 5 and 6: bispecific $F(ab')_2$ (APA03-TSA07) and bispecific $F(ab')_2$ (APA05-TSA07) fragments purified from peak 2 of Fig. 1, respectively; and Lane 7: $F(ab')_2$ of mAb TSA07. Minor bands of 28-30 kDa and 20-22 kDa observed in Lane 2-4 show reduced products of the Fab' fragments, namely truncated heavy chains and intact light chains, respectively.

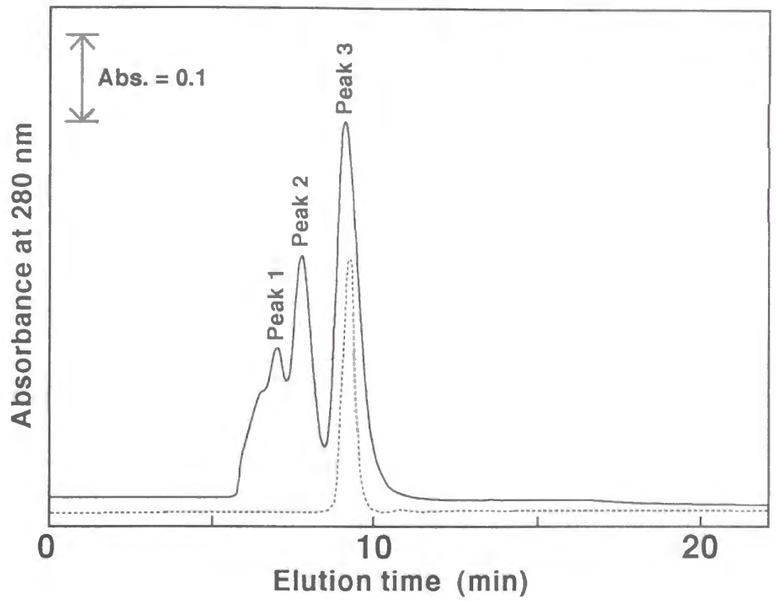

Fig. 3. Purification of polymerized ALP by gel-filtration HPLC on a TSKgel $G3000SW_{XL}$ column. A hundred μl of the polymerization mixture of ALP (containing 1.0 mg protein) was injected to the column (solid line), and 100 μl of the native ALP (0.3 mg) was injected as a control (dotted line).

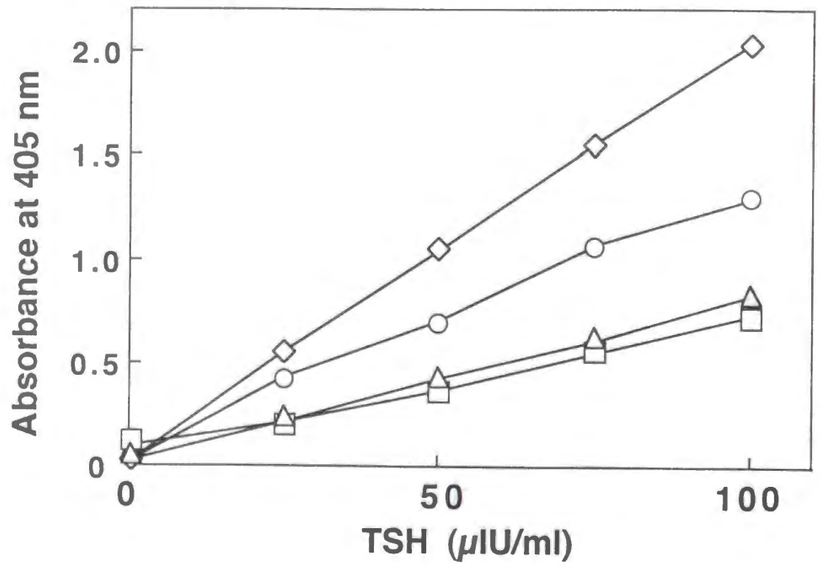

Fig. 4. Immunoreactivities of bispecific $F(ab')_2$ (APA05-TSA07) fragments against TSH. Each well of 96-well microtiter plate was coated with $F(ab')_2$ fragments of anti-TSH mAb (TSA08), and 100 μl TSH solution at the concentration shown in the horizontal axis was added to the well. Then, the plate was incubated with 50 μl of bispecific $F(ab')_2$ (0.75 μg/ml) and 50 μl of the polymerized ALP fractions (the concentration of which was adjusted to 15 μg/ml). Absorbance at 405 nm generated by the reaction with pNPP for 30 min was obtained. Polymerized ALP: peak 1, ◇ ; peak 2, ○; and peak 3, △ . Covalently linked ALP-mAb TSA07 (21 μg/ml) conjugate was shown □ .

than APA03, the bispecific $F(ab')_2$ fragments (APA05-TSA07) was applied to ELISA for TSH. In sandwich assay, the optimal concentration of bispecific $F(ab')_2$ fragments was 0.75 μg/ml (A_{280}=0.001), and those of the covalently linked conjugates of the native ALP with TSA07 mAb conjugate was 21 μg/ml (A_{280}=0.030). The detection limit of TSH was approximately 0.05 μU/ml by using bsmAb and peak 1 of ALP polymer, while 0.15 μU/ml was obtained with using ALP labeled TSA07 mAb conjugate. In Fig. 4, the activity of ALP polymer was more stronger peak 1 than peak 2 and 3. TSH concentration between 0.05 and 100 μU/ml could be measured by one-step new sandwich ELISA.

Discussion

One-step immunoassay using bispecific monoclonal antibody (bsmAb) was first described by Karawajew et al. (25). The calibration curve of antigen using bsmAb was similar to conventional ELISA, and furthermore the low non-specific adsorption of bsmAb is expected to be high sensitivity (19). Recently, homogeneous immunoassay using bsmAb has been developed (26).

Hybrid hybridoma using the cell fusion have been described the extensive chromosomes than hybridoma (6, 9, 27). In addition, heterohybridomas secrete a mixture of monospecific and heterospecific antibodies, and bsmAb theoretically produce about 12.5% of the total immunoglobulins. It is very difficult to purify only bsmAb from ten associated antibodies of the pairs of H and L chains (17).

In this chapter, bispecific $F(ab')_2$ fragments were prepared by the modified method of Brennan et al. (11). The final yields of bispecific $F(ab')_2$ fragments from parental IgG1 were

between 25 and 38%, and the purity were more than 98%. Cook and Wood (28) has described low yield of 7% as chemically preparation method. Most of the losses were as the yield of preparation of $F(ab')_2$ fragments by pepsin digestion. Actually, pepsin digestion is most important procedure to produce the bispecific $F(ab')_2$ fragments. Our procedure of pepsin digestion were suitable and yield much greater quantities (14). Reaction buffer also have to be low pH and the presence of EDTA to avoid the re-oxidation of -SH groups. Furthermore, the bispecific $F(ab')_2$ fragments were clearly purified by hydrophobic interaction HPLC using TSKgel Ether-5PW. These species of antibodies (Fab' fragments of anti-TSH mAb, Fab' fragments of anti-ALP mAb, and bispecific $F(ab')_2$ fragments) were separately eluted in single-step in Fig. 1. The immunoreactivity of bispecific $F(ab')_2$ fragments was only detected in peak 2 fractions. These advantages can lead to a simple and rapid process with high recovery yield.

Bispecific $F(ab')_2$ fragments can theoretically bind both one molecule of antigen and one molecule of enzyme, so it is difficult to increase the enzyme activity against one molecule of antigen. Thus, most of the groups have proved that one-step ELISA using bsmAb gave almost the same sensitivity as ELISA using enzyme-mAb conjugates (9, 25, 17) or recombinant antibody-alkaline phosphatase conjugates (29). The author has developed the new one-step sandwich ELISA method using bispecific $F(ab')_2$ fragments and ALP polymer (Fig. 5). Fig. 5A showed one-step sandwich using ALP-mAb conjugates, and Fig. 5B showed one-step ELISA using both bispecific $F(ab')_2$ fragments and native enzyme. Fig. 5C showed that new ELISA method revealed markedly increase the sensitivity than the other ELISA method. In fact, the sensitivity of ELISA has evidently increase the degree of polymerization of ALP (Fig. 4). In Fig. 4, the combination of peak 1 and bispecific $F(ab')_2$ fragments showed detection limit of 0.05 μU/ml TSH, and three times

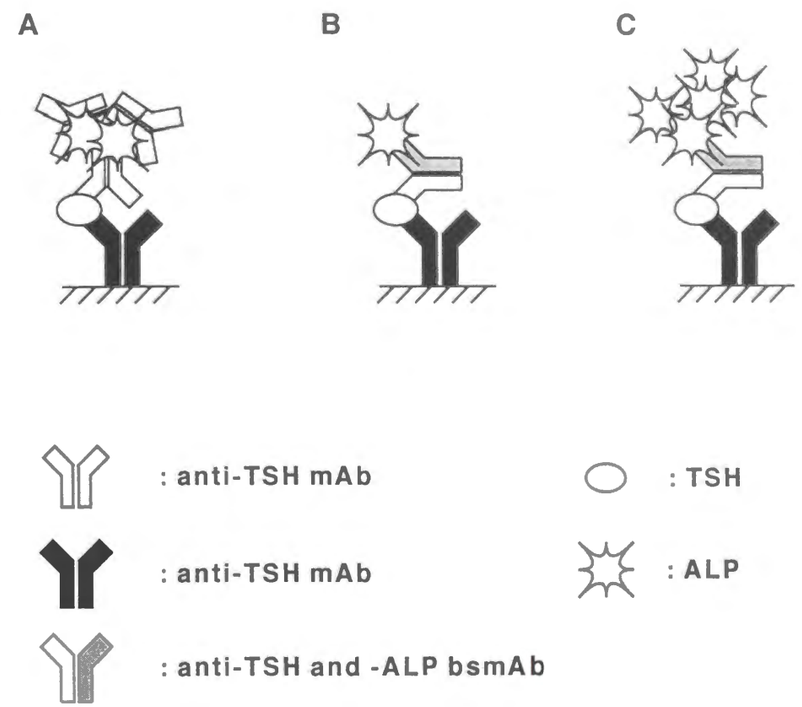

Fig. 5. Schematic representation of various sandwich ELISA model. A shows a sandwich model using enzyme-mAb conjugate, B shows using bsmAb and enzyme, C shows using bsmAb and polymerized enzyme.

sensitivity than the covalently linked ALP-F(ab')$_2$ (TSA07) conjugate. Furthermore, enzyme-mAb may have been some losses of immunoreactivity and enzyme activity. This assay took for one hour in one-step incubation. Moreover, using new chemiluminescent substrates for ALP can improve the sensitivity of ELISA (30-32).

In conclusion, the author has demonstrated that the combination with bsmAb and enzyme polymer can offer many advantages to sandwich ELISA system. The entire assay is performed highly sensitivity and reproducibility with a simple protocol.

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Chapter 4

Effects of Blocking Conditions with Bovine Albumin on the Sensitivity of Enzyme Immunoassay

Introduction

Monoclonal antibody (mAb) is easy to be prepared (1) and widely utilized for detection of its antigen. mAb is used extensively as biochemical reagents of high specificity and affinity in many fields. Especially, solid-phase sandwich enzyme-linked immunosorbent assay (sELISA) is one of the most useful for both basic research and immunodiagnosis (2). In sELISA, mAbs is immobilized on the solid and the solid is blocked with non-reacting proteins (e. g., BSA, gelatin, and casein). Albumin can bind to the surfaces of glasses, plastics, metals or polymers (3, 4). Antibody immobilized and blocking with BSA for sELISA previously described have been generally at neutral pH. On the other hand, mAbs are immobilized on the solid such as polystyrene by passive adsorption. Therefore, it is difficult to control orientation of the mAb molecule on the surface to be more accessible to antigen, and so that immobilized mAb may have enough potential to bind more antigens as possible (5, 6). Solid phase should be hydrophilic and could bind all kinds of antigen or antibody in natural pH. Covalent immobilization of biomolecules was reported in highly hydrophilic surroundings readily recognized many peptides (7, 8).

To optimize the conditions of BSA blocking for improvement of the sensitivity of sELISA, acidic buffer was examined as well as a variety of blocking buffers. Citrate buffer

is shown to be more suitable to block nonspecific absorption onto the surfaces, and it enhances the antigen binding ability of immobilized mAbs.

Materials and methods

Monoclonal Antibodies

Three $F(ab')_2$ fragments, an IgG1 mAb, and an IgM mAbs used were I54N, 28BK, 31CB, T51F, and 23AC, respectively. Their specific antigen is human myoglobin. The hybridomas secreting these mAbs were established in our laboratory according to Köhler and Milstein (1). The mAbs were purified from the ascites by 60% ammonium sulfate saturation followed by gel filtration on TSKgel Toyopearl HW-55 (Tosoh, Tokyo, Japan) in 100 mM sodium citrate buffer, pH 4.5 (9). The $F(ab')_2$ fragments of I54N, 28BK, and 31CB were prepared by the method of Morimoto and Inouye (10). BSA (Cohn fraction V) was purchased from Sigma (St. Louis, MO).

Sandwich enzyme linked immunosorbent assay

Magnetic particles were coated with 100 μl of mouse anti-myoglobin mAb (IgG1, $F(ab')_2$, IgM) (5 μg/ml in 50 mM Tris-HCl, pH 8.0) in a tube, followed by incubation for 30 min at 25°C. Magnetic particles were blocked by incubation with 0.1% (w/v) BSA in various conditions (e. g., incubation time, pH, and buffer component) at 25°C. Blocking time was monitored at 0, 3, 30, 60, 120, 240, 360, and 720 min. pH of blocking buffer was adjusted to 2.5-8.5. Tris-HCl, phosphate, acetate, glycine-HCl, phthalate, and citrate buffers (100 mM) were used for the blocking buffers. After washing the particle with PBS, 100 μl of

human myoglobin (0, and 250 ng/ml) (Scrrips Laboratories, San Diego, CA) and 100 μl of goat anti-myoglobin polyclonal antibody conjugated with alkaline phosphatase (1/5,000; Jackson Immunoresearch Laboratories, West Grove, PA) were added to the tube and incubated for 1 h at 25°C. The particle were washed with PBS, followed by addition of 200 μl of the substrate (3.0 mg/ml pNPP in 50 mM carbonate buffer, pH 9.5 containing 10 mM $MgCl_2$) for 3 min at 25°C. The reaction was terminated by adding 200 μl of 1 N NaOH, and each solution was measured absorbance at 405 nm.

Hydrophobic interaction HPLC

The HPLC apparatus composed of a solvent-delivery system CCPM, a UV monitoring system UV8010, a fraction collector FC8000 and a computer control system SC8010 was purchased from Tosoh. The elution was monitored by absorbance at 280 nm, and each fraction (1 ml) were collected.

Hydrophobic interaction HPLC was performed on a TSKgel Ether-5PW column (7.5 mm (inner diameter) x 75 mm) (Tosoh). mAbs were dialyzed against PBS (pH 7.4) or citrate (pH 3.5) containing 1.5 M ammonium sulfate for 2 h at 4°C. The solution was applied to the column equilibrated with the same buffer, and eluted with a linear gradient of ammonium sulfate from 2 to 0 M, for 30 min at a flow-rate of 1 ml/min at room temperature.

Results and discussion

BSA is a major protein in blood plasma consists of 583 amino acid residues in blood plasma, and more than 30% of the amino acids are charged residues. It is known that BSA

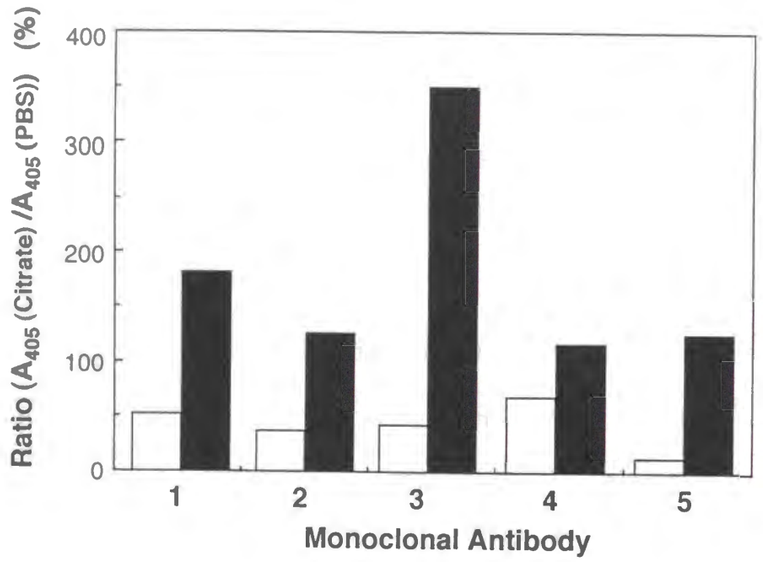

Fig. 1. Ratio of the immunoreactivities of mAbs with 100 mM citrate at pH 3.5 or PBS at pH 7.4 blocking buffer. Each magnetic particles were coated with mAb (1, I54N, $F(ab')_2$; 2, 28BK, $F(ab')_2$; 3, 31CB, $F(ab')_2$; 4, T51F, IgG1; 5, 23AC, IgM). Blocking solution were 100 mM citrate at pH 3.5 or PBS at pH 7.4 containing 0.1% BSA. Vertical axis was the percentage of the immunoreactivity of mAbs using PBS blocking buffer. Absorbance at 405 nm was obtained by the reaction with pNPP for 30 min at 25°C. Standard solution of myoglobin: 0 ng/ml, □ ; 250 ng/ml, ■ .

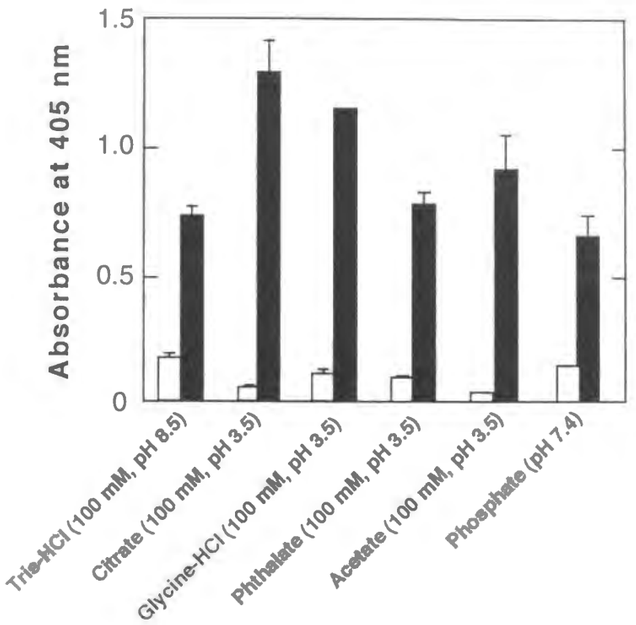

Fig. 2. The immunoreactivities of I54N $F(ab')_2$ fragments with various blocking buffers. Each magnetic particles were coated with $F(ab')_2$ fragments of anti-myoglobin mAb (I54N), and 300 μl of blocking solution at various buffers containing 0.1% BSA shown in the horizontal axis was added. Then, the magnetic particles were incubated with 50 μl of anti-myoglobin polyclonal antibody conjugated with alkaline phosphatase and 50 μl of standard solution. Absorbance at 405 nm was obtained by the reaction with pNPP for 30 min at 25°C. Standard solution of myoglobin: 0 ng/ml, □ ; 250 ng/ml, ■ .

Table 1 Elution time of hydrophobic interaction HPLC using TSKgel Phenyl-5PW

Monoclonal Antibody		Elution time (min) [a]	
		PBS (pH 7.4)	Citrate (pH 3.5)
I54N	$F(ab')_2$	33.23	34.55
28BK	$F(ab')_2$	26.06	26.23
31CB	$F(ab')_2$	25.47	30.20
T51F	IgG1	22.57	23.17
23AC	IgM	23.22	23.39

[a] Elution was monitored at a flow-rate of 1 ml/min by absorbance at 280 nm.

is irreversibly denatured at temperature above 50°C and occurs the acid-induced conformational expansion (11). BSA is generally used as a blocking agent preventing nonspecific absorption to the solid in ELISA. Blocking the solid phase with BSA has been usually done under neutral pH conditions without precise examination.

In Figure 1, citrate buffer (pH 3.5) was shown more suitable to block BSA onto the surfaces than phosphate buffer (pH 7.4). Furthermore, a significant increase in absorbance at 250 ng/ml of human myoglobin was observed in acidic pH, suggesting that antigen-accessibility may cause steric hinderance or destruction of epitopes in neutral pH (6). Surprisingly, the citrate buffer (pH 3.5) strongly activated antigen binding ability of mAb in comparison with other buffers (Fig. 2). Generally, the biomolecules are simply immobilized on the magnetic particles by passive adsorption with hydrophobic interactions. Citrate buffer (pH 3.5) may change hydrophobic surroundings of the immobilized mAb (Table 1), and reorientate themselves to access the antigen. Adsorption conditions of mAb were shown to be important when mAb immobilized on the surfaces of solid.

Furthermore, the other advantage of the citrate buffer in sELISA was shorten the blocking time to 3 min. In the conventional neutral buffers, it has been 60 min. This improvement was suggested to due to denature of BSA conformation (11).

In conclusion, it was demonstrated that 100 mM citrate buffer (pH 3.5) in the BSA blocking can offer advantages to sandwich ELISA system. The entire assay is performed with sensitivity and reproducibility. The method described here is recommended for the improvement in the sensitivity of sELISA.

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Summary

Chapter 1

Section 1

Hydrophobic interaction high performance liquid chromatography (HPLC) using TSKgel Phenyl-5PW was applicable to single-step purification of $F(ab')_2$ fragments from pepsin-digests of mouse mAbs of IgG1 class. The digests were applied to the gel equilibrated with phosphate buffered saline containing 1 M ammonium sulfate. $F(ab')_2$ fragments were adsorbed onto the gel using the same buffer, and eluted by reducing the ammonium sulfate concentration to 0 M. The fraction containing $F(ab')_2$ fragments was homogeneous (purity: higher than 98%) by both SDS-PAGE and gel filtration HPLC. The recovery of the antigen binding site was 42-58%. The cycle time of the Phenyl-5PW HPLC was 45 min, and $F(ab')_2$ fragments of up to 2200 mg was purified in a cycle. This method could be useful especially for large-scale purification of $F(ab')_2$ fragments.

Section 2

A procedure is described for preparation and single-step purification of $F(ab')_2$ fragments, herein designated as $F(ab')_{2\mu}$ from mouse mAbs of the IgM class. HPLC using TSKgel Ether-5PW was well applicable to the purification. The IgM was digested with pepsin at the pepsin-to-IgM ratio of 1:200 (w/w) in 100 mM citrate buffer (pH 4.2) at 37°C for 2 h. The digests were applied to the gel equilibrated with the buffer containing 1 M ammonium sulfate. $F(ab')_{2\mu}$ fragments were adsorbed onto the gel with the same buffer, and eluted by reducing the ammonium sulfate concentration to 0 M. The fraction containing $F(ab')_{2\mu}$

fragments was homogeneous (purity higher than 97%) by both SDS-PAGE and gel filtration HPLC. The recovery of the antigen-binding site was 55-72%. The cycle time of the Ether-5PW HPLC was 40 min, and up to 98 mg $F(ab')_{2\mu}$ fragments was purified in a cycle. This method could be suitable especially for large-scale purification of $F(ab')_{2\mu}$ fragments. The molecular mass of $F(ab')_{2\mu}$ fragments was estimated to be 144-146 kDa. In comparison with IgM, $F(ab')_{2\mu}$ lost entirely the complement C1q binding activity, and the sugar content was greatly reduced. The binding of IgM with non-specific proteins turned to be negligible, when IgM was converted to $F(ab')_{2\mu}$ fragments. The author also showed that the $F(ab')_{2\mu}$ was superior to the IgM as fluorescent staining antibodies to cell surface antigen in flow cytometric analysis. These results suggest that the $F(ab')_{2\mu}$ fragments against the surface marker was readily available for immunological application.

Section 3

A preparation and application of rat $F(ab')_{2\mu}$ fragments against murine interleukin-6 were described. The rat IgM monoclonal antibodies could be sufficiently digested at a pepsin-to IgM ratio of 1:200 (w/w) in 100 mM citrate buffer (pH 4.5) at 37°C for 2 h. Four $F(ab')_{2\mu}$ were homogeneous of a molecular mass of 147-153 kDa consisted L-chain (27 kDa) and truncated H-chain (44 and 48 kDa), and a 1:1 ratio of heavy and light chains. The author found that hydrophobic interaction HPLC with TSKgel Phenyl-5PW provided excellent resolution of $F(ab')_{2\mu}$ in a single step, and $F(ab')_{2\mu}$ were obtained more than 94% purity and 41-52% recovery yield. The immunoreactivity of $F(ab')_{2\mu}$ were entirely maintained. Using $F(ab')_{2\mu}$ for sandwich enzyme immunoassay of mIL-6, sensitivity and reproducibility were greatly improved. $F(ab')_{2\mu}$ could be more useful antibody than IgM as immunological reagent.

Chapter 2

$F(ab')_2$ fragments, herein designated as $F(ab')_{2\mu}$ fragments, were prepared from a mouse IgM monoclonal antibody specific to sialyl Lewis ^{a} antigen. The $F(ab')_{2\mu}$ fragments were applied to flow cytometry to analyze the antigen on human cancer cell surface. The binding of the $F(ab')_{2\mu}$ fragments to the antigen-positive cells (Colo201 and SW1116) was stronger than that of the original IgM. The non-specific binding of the IgM monoclonal antibody to the antigen-negative cells (Capan2, MKN74, PC9, and QG56) was much decreased by using the $F(ab')_{2\mu}$ fragments. These results indicate that the $F(ab')_{2\mu}$ fragments are more suitable than the original IgM monoclonal antibody in flow cytometric analysis.

Chapter 3

Bispecific $F(ab')_{2\mu}$ fragments, recognizing both human stimulating hormone (TSH) and calf intestine alkaline phosphatase (ALP), were prepared by chemical linkage method, and were clearly purified Fab' fragments of no-reactive bispecific activity by hydrophobic interaction HPLC with TSKgel Ether-5PW. The final yield of bsmAb from parental IgG were estimated 25-38%, and purity were more than 98%. ALP was mildly polymerized by glutaraldehyde and fractionated by gel-filtration HPLC. A new sandwich ELISA for TSH was developed by using the ALP polymers and bispecific $F(ab')_2$ fragments against TSH and ALP. In this assay, preparation of covalently linked enzyme-mAb conjugates is not needed. These sensitivity for TSH increases in proportion to the degree of polymerization of ALP, and the lower detection limit obtained 0.05 μU/ml. The sensitivity is thirty times or more higher than that by the conventional sandwich ELISA using covalently linked

enzyme-mAb conjugates. ELISA using both enzyme polymers and bispecific $F(ab')_2$ might be generally applicable for other antigens.

Chapter 4

An improved method is described for a sandwich enzyme linked immunosorbent assay (sELISA). Magnetic particles immobilized with IgG1, $F(ab')_2$, or IgM mAbs were blocked with BSA under acidic conditions (100 mM citrate buffer, pH 3.5, 3 min). The blocking conditions represent a significant improvement in sensitivity in sELISA in comparison with the previously described method. This improvement is resulted from increased binding of mAbs to the antigen by an increased antigen-binding capacity of immobilized mAbs. The conditions appear to provide optimal orientation of the antigen binding sites of mAbs, and immobilized mAbs may access to the antigen in a more suitable manner.

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List of Publications

Koichi Morimoto and Kuniyo Inouye

Single-step purification of $F(ab')_2$ fragments of mouse monoclonal antibodies (immunoglobulins G1) by hydrophobic interaction high performance liquid chromatography using TSKgel Phenyl-5PW.

Journal of Biochemical and Biophysical Methods **24**, 107-117 (1992).

Kuniyo Inouye and Koichi Morimoto

Single-step purification of $F(ab')_{2\mu}$ fragments of mouse monoclonal antibodies (immunoglobulins M) by hydrophobic interaction high-performance liquid chromatography using TSKgel Ether-5PW.

Journal of Biochemical and Biophysical Methods **26**, 27-39 (1993).

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Preparation of $F(ab')_{2\mu}$ fragments from rat IgM monoclonal antibodies and their application to the enzyme immunoassay of mouse interleukin-6.

Journal of Immunological Methods **171**, 239-244 (1994).

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Flow cytometric analysis of sialyl Lewis ^{a} antigen on human cancer cells by using $F(ab')_{2\mu}$ fragments prepared from a mouse IgM monoclonal antibody.

Cytotechnology (1997) in press.

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A sensitive enzyme immunoassay of thyroid-stimulating hormone (TSH) by using bispecific $F(ab')_2$ fragments recognizing polymerized alkaline phosphatase and TSH.

submitted to *Journal of Immunological Methods*.

Koichi Morimoto and Kuniyo Inouye

Effects of blocking conditions with bovine albumin on the sensitivity of enzyme immunoassay. in preparation.